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(71) Applicant : ELI LILLY AND COMPANY
Lilly Corporate Center
Indianapolis Indiana 46285 (US)

(72) Inventor : Kreusman, Adam Joseph
3630 Redwood Drive
Indianapolis, Indiana 46227 (US)
Inventor : Yeh, Wu-Kuang
720 Primrose Court
Greenwood, Indiana 46142 (US)

(74) Representative : Tapping, Kenneth George et al
Erl Wood Manor
Windlesham Surrey, GU20 6PH (GB)

(54) Lipopeptide deacylase.

(57) Echinocandin B deacylase, a cell-associated enzyme from Actinoplanes utahensis, is purified to near homogeneity in a process comprising hydrophobic interaction chromatography, cation-exchange chromatography and gel filtrations. The enzyme is a heterodimer containing 18-KD and 63-KD subunits and is a simple enzyme unaffected in activity by co-factors, metal chelators, or sulfhydryl reagents. The enzyme catalyzes the deacylation of the lipid acyl portion of lipid cyclicpeptide metabolites such as ECB and aculeacin.

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Jouve, 18, rue Saint-Denis, 75001 PARIS

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This invention relates to enzyme technology. In particular it relates to a lipopeptide deacylase in purified form produced by the organism Actinoplanes utahensis which deacylates the lipophilic acyl side chains of the antifungal metabolites echinocandin B (ECB), aculeacin, and analogs of ECB.

Echinocandin B and aculeacin are known cyclic hexapeptides having the linoleoyl and palmitoyl side chains respectively. Boeck, L. D., et al., 1988. J. Antibiot. (Tokyo), 41, 1085-1092; Boeck, L. D., et al., 1989 J. Antibiot (Tokyo), 42, 382-388; and Kimura, Y., et al., 1987. Agri. Biol. Chem. 51, 1617-1623; report the deacylation of the linoleoyl group of ECB with whole cells of Actinoplanes utahensis and Pseudomonas species. Takeshima, H., et al., 1989. J. Biochem. 105, 606-610, report the purification and partial characterization of a deacylase from A. utahensis which deacylates aculeacin.

Structural modification of the natural antifungals has led to potentially useful therapeutic agents such as cilofungin and daptomycin. Debono, M., et al., 1989. J. Antibiot. (Tokyo), 42, 389-397; Debono, M., et al., 1988. Ann. N.Y. Acad. Sci. 544, 152-167; Gordee, R.S. et al., 1988. Ann. N.Y. Acad. Sci. 544, 294-309; and Boeck, L.D., et al., 1988. J. Antibiot. (Tokyo), 41, 1085-1092. For example, ECB has been deacylated with whole cells of A. utahensis to cleave the lipophilic acyl side chain to provide the cyclic hexapeptide nucleus of ECB. Reacylation of the nucleus by chemical means has provided the acyl ECB compounds such as cilofungin with improved antifungal prperties.

Because of the need for improved antifungal agents for the treatment of systemic fungal infections methods for their production are important. Enzymatic methods for preparing such compounds are especially desirable since they are usually simpler and more economical methods than chemical methods. Accordingly the availability of enzymes useful for such conversions is highly desirable.

Actinoplanes utahensis deacylase is provided in purified form. The enzyme catalyzes the cleavage of the linoleoyl group of ECB and the palmitoyl group of aculeacin. The enzyme is a heterodimer comprising 63 KD and 18 KD subunits which is optimally active at pH 6.0 and 60° C. The enzyme is cell associated and is not affected by cofactors, metal ion chelators or sulfhydryl reagents.

The enzyme is useful in a method for the preparation of cyclic hexapeptide nuclei and in a method for the preparation of the cyclicpeptide nucleus of the A21978C antibiotics.

The invention also provides a method for purifying the deacylase enzyme to near homogeneity which comprises hydrophobic interaction, cation-exchange, dye-ligand chromatographies and gel filtrations.

The deacylase of Actinoplanes utahensis provided by this invention is referred to herein for convenience as ECB deacylase. The enzyme all of which is virtually cell-associated has the following physical, catalytic and kinetic properties in its purified state.

ECB deacylase is an 81-kilodalton (KD) heterodimer comprising 63-KD and 18 KD subunits. The amino-terminal sequences of the large and small subunits are respectively as follows.

Ser-Asn-Ala-Tyr-Gly-Leu-Gly-Ala-Gln-Ala-Thr-
Val-Asn-Gly-Ser-Gly-Met-Val-Leu-Ala-Asn-Pro-
His-Phe-Pro-(Trp)-Gln --- Ala-Glu-(Arg)-Phe-Tyr.

His-Asp-Gly-Gly-Tyr-Ala-Ala-Leu-Ile-Arg-Arg-Ala-
Ser-Tyr-Gly-Val-(Pro)-His-Ile-Thr-Ala-Asp-Asp-Phe.

In the above sequences the amino acid residues in parentheses indicate a tentative assignment while "---" indicates that the residue has not as yet been identified.

The amino acid composition of the purified enzyme is shown below in Table I. The composition was determined by the method described by Dotzlaf, J. E. and Yeh, W-K, 1987. J. Bacteriol. 169, 1611-1618.

TABLE I
Amino Acid Composition of
A. utahensis Deacylase

Amino Acid	Number of Residues per 81,000-dalton
Asp+Asn	74
Thr	51[a]
Ser	83[a]
Glu+Gln	45
Pro	42
Gly	85
Ala	79
Cys	10[b]
Val	48
Met	5
Ile	25
Leu	53
Tyr	20
Phe	24
His	21
Lys	11
Arg	62
Trp	19[c]

[a] Determined by extrapolation to zero time of hydrolysis.

[b] Determined by cysteic acid.

[c] Determined by hydrolysis in the presence of thioglycolic acid.

The molecular weight of ECB deacylase as estimated by gel filtration with Ultrogel AcA 44 was 46,000. The molecular weights of the subunits described above was determined by SDS-PAGE. The molecular weight determined by gel filtration is a significant underestimate that can be attributed to an abnormal gel elution behavior of the enzyme. The abnormal elution behavior of the enzyme on gel is typical of a membrane-bound protein attributable to the usually high hydrophobicity of the macromolecule.

ECB deacylase is a simple enzyme (although containing two subunits) that does not require any exogenous phospholipid, cofactor, metal ion or reducing agent for expression of its activity. Further, none of the common cofactors, metal ions or reducing agents stimulate the deacylase. Surprisingly, N-ethylmaleimide (NEM) enhances the rate of conversion of ECB to ECB nucleus about 6-7 fold and the purified deacylase.

An important property of the purified deacylase is its enhanced activity in the presence of high salt concentrations. The activity is increased by up to 3-fold in the presence of several common mono-and divalent metal salts. Salts such as sodium chloride, potassium chloride, magnesium chloride, calcium chloride, sodium or potassium nitrate are among such stimulatory salts. A preferred salt for this purpose is potassium chlorid .

Salt concentrations of about 0.1 molar up to about 3.0 molar appear to be the most favorable. Enhanced activity is observed at lower salt concentrations.

The lack of resolution observed for the deacylase by native-PAGE at pH 7 and 9 indicates that the isoelectric point of the enzyme is above 9. The native-PAGE was performed according to Blackshear, P. J. (1984) Methods Enzymol. 104, 237-255.

The substrate employed for study of the kinetic and catalytic properties of the purified enzyme was echinocandin B (ECB). ECB is essentially insoluble in aqueous solutions. Among several water miscible solvents tested for solubilization of ECB in aqueous media, dimethylsulfoxide (DMSO) at a low concentration of about 15% or lower was most compatible with the deacylase catalyzed reaction and subsequent enzyme activity analysis of HPLC.

The enzyme is optimally active at pH 6.0 at 60° C in 0.05M KH_2PO_4 buffer. It was found that the enzyme was at least twice as active when the reaction (ECB to ECB nucleus) was initiated with the enzyme rather than with the substrate.

The Km of the deacylase for echinocandin B, as determined by the Lineweaver-Burk method, was 50 μM.

The Vmax for the ECB reaction was 10-11 μmol of the peptide (ECB nucleus) formed/min/mg protein.

Regarding the reaction stoichiometry, the ratio of ECB nucleus formed to ECB disappearance was observed to be about 52.4%. The low ratio of conversion observed may be attributable to the occurrence of some other reaction or possibly to some degradation.

As was noted hereinabove the A. utahensis deacylase is cell-associated. For example, in a typical 90-hour culture broth of A. utahensis that exhibits a high total activity of ECB deacylase, over 99% of the deacylase activity was cell-associated. Less than 5% of the cell-associated deacylase activity was released by incubation of the cells in 0.01M KH_2PO_4, pH 6.0 for one day. Increase of the ionic strength of this and other buffers had only a slight effect in recovery of a soluble deacylase. However it has been found that by treating the cells with salts such as potassium or sodium chloride, potassium or sodium nitrate and magnesium or calcium salts, a high recovery (60 to 80%) of soluble deacylase is realized. The effect of this salt-treatment is shown in Table II below.

Table II

Solubilization of ECB Deacylase From A. Utahensis

Cell Treatment[a]	Activity Distribution (%)	Specific Activity (U/mg protein)
KCl/KH_2PO_4 and Sonication		
Supernatant Fraction	80	0.1
Pellet Fraction	20	-
KCl/KH_2PO_4 Only		
Supernatant Fraction	60-80	0.2-0.4
Pellet Fraction	20-40	-
KH_2PO_4 and Sonication		
Supernatant Fraction	80	0.1
Pellet Fraction	20	-
KH_2PO_4 Only		
Supernatant Fraction	0	-
Pellet Fraction	100	-

[a] A. utahensis cells were resuspended in 0.8 M KCl/0.05M KH_2PO_4 or 0.05M KH_2PO_4 only, pH 6.0, sonicated continually for one minute, wherever specified, and centrifuted at 48,000 x g for one hour.

The ECB deacylase is produced by culturing Actinoplanes utahensis under submerged aerobic fermentation conditions. The fermentation method and the conditions employed are known, Boeck, L. D., Fukuda, D. S., Abbott, B. J. and Debono, M., 1989. J. Antibiot. (Tokyo), 42, 382-388. Maximum production of the deacylase activity occurs at about 90 hours after inoculation of the culture medium. As described above and in Example 1 hereinafter the enzyme in crude form is solubilized by salt treatment of the whole cells preferably with potassium chloride.

The crude solubilized enzyme is purified to near homogenity in the purification process of the invention which comprises hydrophobic interaction chromatography, cation-exchange chromatography and gel filtration steps. As described above herein the ECB deacylase behaves as a loosely cell-bound protein and is solubilized differentially by treating whole cells of A. utahensis with an inorganic salt. Preferably potassium chloride is used. The solubilized enzyme solution is desirably filtered and the filtrate concentrated by ultrafiltration to provide a more concentrated deacylase solution for the ensuing steps.

In the first step of the process the concentrated cell extract is heated for one hour at about 60° C and is treated while warm with ammonium sulfate, 14%, and potassium chloride, 1.2M. The heat treatment causes other proteins in the solution which are unstable at 60° C to precipitate. The ECB deacylase is stable at 60° C and remains solubilized.

The heat treated extract is then subjected to hydrophobic interaction chromatography at a temperature of about 25° C on a hydrophobic interaction chromatographic material such as lipid substituted agarose for example the commercially available Octyl Sepharose (Pharmacia). The column is equilibrated to pH 6 with about 0.05M potassium dihydrogen phosphate or other suitable buffer, 1.2M potassium chloride and 14% ammonium sulfate. The column is desirably washed with the same buffer and the bound protein is eluted with a simultaneous linear gradient of KCl (1.2-0.1M) and $(NH_4)_2SO_4$ (14-0%) in 0.05M KH_2PO_4, pH 6 buffer. The ECB deacylase is eluted as a single, unsymmetrical activity peak.

The peak fractions containing about 95% of the total deacylase activity are pooled and subj cted to ammonium sulfate fractionation. The 10-36% $(NH_4)_2SO_4$ fraction is collected and subjected to gel filtration in

pH 6 buffer containing 0.8M KCl. Gel types which can be used may vary however Sephacryl S-200 HR is a suitable gel. The enzyme is eluted as one main activity peak and a minor activity peak. The peak fractions which contain 90% of the deacylase activity from the main peak are pooled and adjusted to 0.05 M KCl at pH 5.6. The pooled fractions are subjected to cation-exchange chromatography over a suitable cation-exchange resin such as one of the commercially available Trisacryl resins for example, Trisacryl-CM (IBF Biotechnics). The column is equilibrated with 0.05M KH_2PO_4, pH 5.6, and 0.05M KCl. The column is washed with the same buffer and the bound proteins are eluted with a linear gradient of KCl (0.05M - 0.5M) in the same buffer. The deacylase enzyme is eluted as two activity peaks.

The fractions containing the deacylase activity from each of the peaks are pooled, adjusted to 0.05M KCl, and applied to a dye-ligand gel such as Red-Sepharose (Pharmacia, Inc.) or Dyematrex Blue A (Amicon). The gel is equilibrated prior to use with 0.05M KH_2PO_4, pH 6.0, and 0.05M KCl. The bound proteins are eluted preferably with a step-wise gradient of KCl (0.05-2-3.3M) in the same buffer from the Red-Sepharose or preferably with a linear gradient of KCl (0.05 - 2.5M) in the same buffer from the Blue A gel. A broad and unsymmetrical activity peak is obtained from the dye-ligand chromatography.

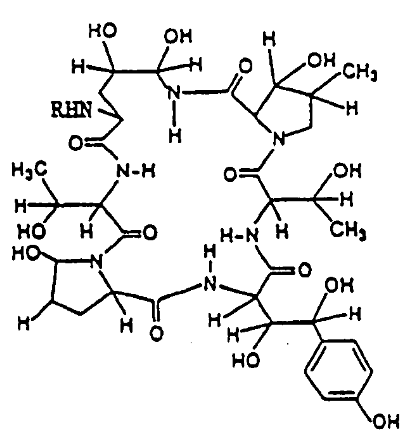
The peak fractions containing about 80% of the total activity from the dye-ligand gel are pooled and subjected to gel filtration over a gel of the type such as Ultragel AcA 44 (IBF Biotechnics) previously equilibrated with 0.05M KH_2PO_4, pH 6.0, and 0.2M KCl. The enzyme is eluted as a single activity peak and the peak fractions containing all of the deacylase are pooled.

The pooled fractions are adjusted to 0.04M KCl at pH 7.0 and then again subjected to cation exchange chromatography over a negatively charged resin such as Trisacryl-CM. The resin is equilibrated prior to use with 0.05M KH_2PO_4, pH 7.0, and 0.04M KCl buffer and bound deacylase is eluted with a step-wise gradient of KCl (0.04 - 0.5 - 2M) in the same buffer. One broad activity peak and one sharp minor activity peak are observed. The fractions from these two activity peaks of the purified enzyme can be stored at -70° C for further use.

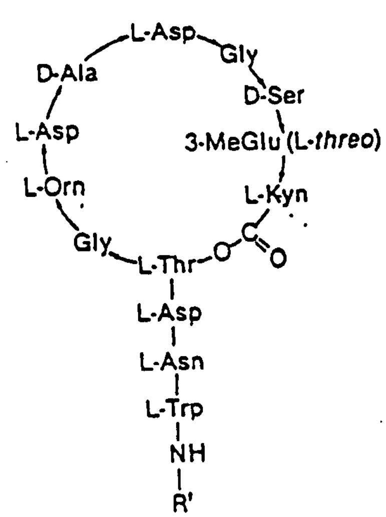
During the foregoing 8-step process for the purification of ECB deacylase two size-forms and two charge-forms of the enzyme are observed. The major peak obtained with the last cation-exchange chromatography was analyzed by SDS-PAGE and showed two closely slow-moving bands and two closely fast-moving bands. The minor cation-exchange chromatography peak showed a single slow-moving band and two fast-moving bands. The absence of the second slow-moving band suggests that this protein is likely a degradation product from the first slow-moving band.

The purified ECB deacylase provided by the purification process of the invention is useful in a method for deacylating lipo cyclicpeptides to provide the cyclicpeptide nuclei thereof. In particular the enzyme deacylates the cyclohexapeptides echinocandin B and aculeacin. Further the purified enzyme cleaves the fatty acid side chain from the cyclicpeptide A21978 antibiotic factors including Daptomycin.

The process of this invention comprises mixing at a temperature between about 25° C and 75° C in an aqueous medium at a pH between about 5 and about 7 a cyclicpeptide represented by the formula A or B



A



B

with echinocandin B deacylase where, in formula A, R is linoleoyl, myristoyl or palmitoyl and, in formula B, R' is decanoyl, 8-methyldecanoyl, 10-methylundecanoyl, or 10-methyldodecanoyl; to provide the compound of the formula A or B wherein R and R' are hydrogen.

The process is preferably carried out at a temperature between about 55° C and about 65° C. The pH of the medium can be maintained with a suitable buffer. A salt such as an alkali metal chloride eg. KCl or NaCl or an alkali metal nitrate eg, KNO3 appears to have a beneficial effect on the activity of the enzyme and can be incorporated in the aqueous medium. Preferably the salt is KCl at a concentration of about 0.05M to about 3.0M.

A water miscible solvent also can be added to the medium in the instance where the substrate's water solubility is low. For example, echinocandin B has a low solubility in water. Dimethylsulfoxide (DMSO) can be added to the reaction medium to enhance its solubility. DMSO is also compatible with the deacylase. In general DMSO can be added in amounts between about 5% and 15% v:v. as demonstrated in the case of ECB and aculeacin.

The process is preferably carried out by forming a solution of the substrate in the buffered aqueous medium, warming the mixture to the reaction temperature and adding the enzyme. The reaction mixture is stirred, shaken or otherwise agitated to provide good contact of substrate and enzyme. The reaction mixture can be monitored from time to time by assaying small aliquots of the mixture by the assay method described hereinafter. Alternatively, the solution of the substrate can be added to the buffered enzyme solution. However, better deacylation results are generally obtained by adding the enzyme to the substrate.

If, as determined by monitoring the reaction, the reaction is proceeding too slowly or has terminated prematurely additional fresh enzyme can be added to complete the deacylation.

The process also may be carried out with an immobilized form of the enzyme. The enzyme may be bonded to a suitable inert resin support and packed into a column. The aqueous buffered solution can be applied to the column and washed through with buffer. Recycling may be required to achieve complete conversion.

A preferred embodiment of the process of the invention comprises the deacylation of echinocandin B (formula A, R = linoleoyl) to the echinocandin B nucleus (formula A, R = H). Another preferred embodiment comprises the deacylation of aculeacin (formula A, R = palmitoyl) to the same ECB nucleus.

The substrate specificity studies carried out with the purified deacylase of the invention revealed rather broad specificity for the cyclicpeptides of the echinocandin B type formula A and the A21978 antibiotic type of formula B. The A21978 substrates are known metabolites described by U.S. Patent No. 4,537,717. Also found to be substrates for the deacylase are certain derivatives of the ECB nucleus which are prepared by the acylation of the nucleus. Examples of such acyl derivatives are represented by the above formula A when R is a 3-phenylpropionyl group substituted in the para position by $C_7H_{15}O$-(30%), $C_6H_{11}O$-(12%); a phenylacetyl group substituted in the para position by C_8H_7O-(30%) or a $C_{11}H_{23}C(O)NH$-group (5%); a benzoyl group substituted in the para position by a $C_{11}H_{23}C(O)NH$-group; or a cinnamoyl group substituted in the para position by a $C_{11}H_{23}C(O)NH$-group (15%). The figures in parentheses are the percent activity relative to the deacylase activity of 100% for ECB itself.

The following example further illustrates and describes the invention but is not intended to be limiting thereof.

Example 1

Preparation and Purification of ECB Deacylase

A. Fermentation of Actinoplanes utahensis Actinoplanes utahensis NRRL 12052 was grown in a 150-liter fermenter under the conditions described by Boeck, L. D., Fukuda, D. S., Abbott, B. J., and Debono, M. 1989. J. Antibiot. (Tokyo), 42, 382-388. Cells containing a high activity of echinocandin B deacylase (90 hours after inoculation) were harvested by centrifugation; washed with 0.05M KH_2PO_4, pH 6.0, and used for enzyme isolation and purification.

B. Enzyme Solubilization and Purification Unless otherwise specified the following isolation and purification procedures were carried out at a temperature between about 0° C and 4° C.

Assay of 100 liters of fermentation medium at 90 hours showed that virtually all of the deacylase activity was cell-associated. The assay method employed throughout this example is described hereinafter.

Fresh cells (7.9 kg wet weight) were re-suspended in 0.05M KH_2PO_4, pH 6.0, with 0.8M KCl to a total volume of 57 liters and the suspension was stirred continuously for one day. Most of the cell-associated deacylase activity became soluble differentially by this salt treatment.

The solubilized deacylase was filtered through Whatman No. 1 paper and the filtrate was concentrated to a volume of 3.3 liters with an Amicon YM 30 spiral ultrafiltration cartridge. The concentrated extract was heated

at 60°C for one hour.

The heat-treated enzyme extract was treated with $(NH_4)_2SO_4$ at 14% concentration and KCl at 1.2M and was loaded onto a Octyl-Sepharose column (5 x 36 cm) previously equilibrated with 0.05 M KH_2PO_4, pH 6.0, 1.2M KCl and 14% $(NH_4)_2SO_4$. The column was washed with two bed volumes of the same buffer and bound proteins were eluted with a simultaneous linear gradient of KCl (1.2-0.1M) and $(NH_4)_2SO_4$ (14-0%) in 0.05M KH_2PO_4, pH 6.0. This chromatography (hydrophobic interaction chromatography) was carried out at a temperature of about 25° C. ECB deacylase was eluted as a single but non-symmetrical activity peak.

The peak fractions containing 95% of the total deacylase activity were pooled and fractionated with $(NH_4)_2SO_4$. The 10-36% $(NH_4)_2SO_4$ fraction was loaded onto a Sephacryl S-200 HR column (5 x 69 cm) previously equilibrated with 0.05M KH_2PO_4, pH 6.0, and 0.8M KCl (buffer A). The deacylase was eluted as a main activity peak and a minor one. The peak fractions containing 90% of the enzyme activity from the main peak were pooled, adjusted to 0.05M KCl at pH 5.6 and were applied to a Triascryl-CM column (2.5 x 33 cm) previously equilibrated with 0.05M KH_2PO_4, pH 5.6, and 0.05M KCl (buffer B). The column was washed with two-bed volumes of buffer B and bound proteins were eluted with a linear gradient of KCl (0.05-0.5M) in buffer B. The deacylase was eluted as two activity peaks.

The fractions containing the deacylase activity from each of the two peaks were pooled, one pool per peak. The two enzyme pools were adjusted to 0.05M KCl and one pool loaded onto a Red-Sepharose column (3.2 x 15.5 cm) and the other onto a Dyematrex Blue A column (2.2 x 22 cm). Both columns were previously equilibrated with 0.05M KH_2PO_4, pH 6.0, and 0.05M KCl (buffer C). After both columns were washed with two bed volumes of buffer C the bound protein was eluted from the Red-Sepharose column with a step-wise gradient of KCl (0.05-2-3.3 M) in buffer C and from the Blue A column with a linear gradient of KCl (0.05-2.5 M) in buffer C. A broad and non-symmetrical activity peak was observed from each dye-ligand chromatography.

The peak fractions containing 80% of the total deacylase activity from the Red-Sepharose peak were pooled and applied to a Ultragel Ac 44 column (1 x 118 cm) previously equilibrated with 0.05 M KH_2PO_4, pH 6.0, and 0.2M KCl (buffer D). The deacylase was eluted as a single activity peak.

The peak fractions containing all of the deacylase activity were pooled, adjusted to 0.04M KCl at pH 7.0 and loaded onto a Trisacryl-CM column (1 x 34 cm) previously equilibrated with 0.05M KH_2PO_4, pH 7.0 and 0.04M KCl (buffer E). After the column was washed with two bed volumes of buffer E bound proteins were eluted with a step-wise gradient of KCl (0.04-0.5-2M) in buffer E. One broad main activity peak and one sharp minor activity peak were observed. The fractions from the two activity peaks were stored individually at -70° C until required.

The following Table III shows the results obtained with each step of the isolation and purification of ECB deacylase detailed above.

Table III

Purification of ECB Deacylase

Step	Protein (mg)	Activity (U)	Sp. Act. (U/mg)	Recovery (%)
Soluble Extract	12,546	4,631	0.37	100
Heat-Treated Extract (60° C for 1-hr)	7,325	3,606	0.49	78
Octyl-Sepharose Eluate	1,357	2,038	1.5	44
10-36% $(NH_4)_2SO_4$ Fraction	1,077	1,513	1.41	33
Sephacryl S-200 HR Eluate	857	1,470	1.72	32
Trisacryl-CM Eluate, pH 5.6 (A)	78	293	3.76	6.3
Trisacryl-CM Eluate, pH 5.6 (B)	175	595	3.42	12.8
Red-Sepharose Eluate (A1)	10.1	58.4	5.78	1.3
Blue A Eluate (B1)	113	368	3.53	7.9
Ultrogel AcA 44 Eluate (A1a)	8.1	60.2	7.42	1.3
Trisacryl-CM Eluate, pH 7.0 (A1a1)	5.5	56.2	10.22	1.2
Trisacryl-CM Eluate, pH 7.0 (A1a2)	1.2	13.5	11.25	0.3

As described hereinabove, two activity peaks were observed in the first and second cation-exchange chromatography with Trisacryl-CM. The fractions from each of the two peaks A and B of the first chromatography were collected and analyzed separately as shown. The pooled A fractions were subjected to the Red-Sepharose dye-ligand chromatography (A1) while those of the second activity peak B were subjected to the Dyematrex Blue A dye-ligand chromatography (B1). When the eluates of each dye-ligand chromatography were analyzed the specific activity increased for the Red-Sepharose treatment while the specific activity for the Blue A increased only slightly. Accordingly, in this instance, the eluate of the Red-Sepharose column was selected for further purification by Ultrogel filtration (A1a) followed by cation-exchange chromatography with Trisacryl-CM. Again, as with the previous cation-exchange chromatography the two activity peaks (enzyme forms) were collected and analyzed separately.

Enzyme assay

The assay employed herein for determining and measuring deacylase activity utilizes echinocandin B as substrate. A typical reaction mixture of 1 ml for ECB deacylase assay contained 425 μmole of ECB and 0.000003 to 0.003 unit of the enzyme in 0.05M KH_2PO_4, pH 6.0, in the presence of 0.68M KCl and 15% DMSO to effect solution of the ECB. The enzymatic reaction was initiated by addition of the enzyme and was continued for 20 min at 60° C before being interrupted by the addition of phosphoric acid. After a low-speed centrifugation to remove precipitated protein, the deacylase activity was determined by monitoring the formation of ECB nucleus at 225 nm using HPLC.

HPLC components were IBM PS/2 Model 80 and Color Display (IBM, Armonk, N.Y.), a Water 715 Ultra WISP Sample Processor (Waters Associates, Milford, MA), and a Beckman System Gold Programmable Solvent Module 126 and Scanning Detector Module 167 (Beckman, Fullerton, CA.).

The ECB nucleus was eluted from an Apex Octadecyl 3 μ column (4.6 x 10 cm) (Jones Chromatography, Littleton, Co.) with a mobile phase of 3.9% CH_3CN/0.1% trifluoroacetic acid and a flow rate of 1 ml/min. The nucleus formation was linear with time during the assays. Duplicated HPLC analyses had an average 2-3% deviation. As used herein one unit of enzyme activity is defined as the amount of the deacylase required to cause formation of one μmole of the ECB nucleus per minute from ECB under the above described reaction conditions.

The specific activity (Table III) is defined as units per mg of protein. The protein content was determined by the method of Bradford using bovine serum albumin as standard (Bradford, M. M., (1976) Anal. Biochem. 72, 248-254.

Claims

1. Echinocandin B deacylase in purified form which is an 81-kilodalton heterodimer having the following amino acid composition:

Amino Acid	Number of Residu s per 81,000-dalton
Asp+Asn	74
Thr	51
Ser	83
Glu+Gln	45
Pro	42
Gly	85
Ala	79
Cys	10
Val	48
Met	5
Ile	25
Leu	53
Tyr	20
Phe	24
His	21
Lys	11
Arg	62
Trp	19

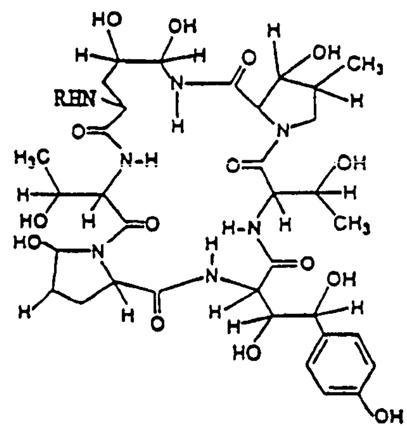
which has a specific activity of between about 10 U/mg and about 11.25 U/mg; which has a 63-kilodalton subunit having the amino-terminal sequence:

Ser-Asn-Ala-Tyr-Gly-Leu-Gly-Ala-Gln-Ala-Thr-Val-Asn-Gly-Ser-Gly-Met-Val-Leu-Ala-Asn-Pro-His-Phe-Pro-(Trp)-Gln --- Ala-Glu-(Arg)-Phe-Tyr; and an 18-kilodalton subunit having the amino-terminal sequence; His-Asp-Gly-Gly-Tyr-Ala-Ala-Leu-Ile-Arg-Arg-Ala-Ser-Tyr-Gly-Val-(Pro)-His-Ile-Thr-Ala-Asp-Asp-Phe; and which in the deacylation of echinocandin B as substrate exhibits optimal catalytic activity at about pH 6.0 at about 60° C in 0.05M KH_2PO_4 buffer.

2. The process for preparing the purified deacylase of claim 1 which comprises the steps, 1) heating for about one hour at a temperature of about 60° C an aqueous solution of crude deacylase buffered at about pH 6.0; 2) chromatographing the heat treated extract buffered at pH 6.0, 1.2M KCl and 14% $(NH_4)_2SO_4$, over an hydrophobic interaction chromatogram; 3) fractionating by $(NH_4)_2SO_4$ the eluate containing 95% of deacylase activity from step 3 and separating the 10% to 36% $(NH_4)_2SO_4$ fraction; 4) gel filtering said $(NH_4)_2SO_4$ fraction in pH 6.0 buffer containing 0.8M KCl and combining the fraction of the eluate containing 90% of the deacylase activity; 5) chromatographing said fractions in a pH 5.6, 0.05M KCl buffer on a cation-exchange chromatogram and eluting said chromatogram with a linear gradient of KCl (0.05-0.5M) in

said buffer; 6) adjusting the deacylase containing the eluate of step 5 to 0.05M KCl, chromatographing said eluate on a dye-ligand chromatogram in 0.05M KH_2PO_4, pH 6.0, and 0.05M KCl buffer and eluting said chromatogram with a linear gradient of KCl (0.05M-2.5M); 7) gel filtering the pooled eluate fractions from step 6 containing 80% of the deacylase activity in a 0.05M KH_2PO_4, pH 6.0, and 0.2M KCl buffer; and 8) adjusting to 0.04M KCl at pH 7 the deacylase containing eluate of step 7, chromatographing said eluate on a cation-exchange chromatogram in 0.05M KH_2PO_4, pH 7.0, and 0.04M KCl buffer and eluting the purified deacylase with a step-wise gradient of KCl (0.04-0.5-2M).

3. The process for deacylating a cyclicpeptide of the formula A or B



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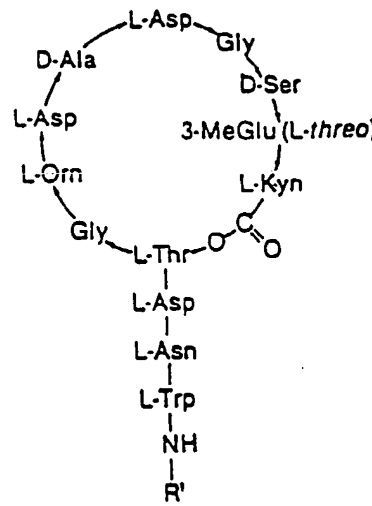


B

which comprises mixing said cyclicpeptide at a temperature between about 25° C and about 75° C in an aqueous medium at a pH between about 5 and about 7 with the purified deacylase of claim 1 where, in formula A, R is linoleoyl, myristoyl or palmitoyl and, in formula B, R′ is decanoyl, 8-methyldecanoyl, 10-methyl-undecanoyl or 10-methyldodecanoyl; to provide the compound of the formula A or B wherein R or R′ is hydrogen.

4. The process of claim 3 wherein the temperature is maintained at about 55° C to about 65° C.

5. The process of claim 3 wherein the aqueous medium contains an inorganic salt selected from an alkali metal chloride or an alkali metal nitrate.

6. The process of claim 5 wherein the salt is potassium chloride at a concentration of between about 0.1M to about 3.0M.

7. The process of claim 3 where, in formula A, R is linoleoyl.

8. The process of claim 3 where, in formula A, R is palmitoyl.

9. The process of claim 3 where, in formula B, R′ is decanoyl.

10. The process of claim 3 wherein the aqueous medium contains dimethylsulfoxide at a concentration between about 5% and about 15%.

Claims f r the following C ntracting States: ES

1. The process for preparing Echinocandin B deacylase in purified form which is an 81-kilodalton heterodimer

having the following amino acid composition:

Amino Acid	Number of Residues per 81,000-dalton
Asp+Asn	74
Thr	51
Ser	83
Glu+Gln	45
Pro	42
Gly	85
Ala	79
Cys	10
Val	48
Met	5
Ile	25
Leu	53
Tyr	20
Phe	24
His	21
Lys	11
Arg	62
Trp	19

which has a specific activity of between about 10 U/mg and about 11.25 U/mg; which has a 63-kilodalton subunit having the amino-terminal sequence:

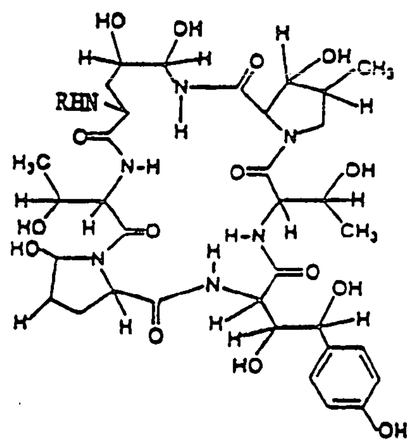
Ser-Asn-Ala-Tyr-Gly-Leu-Gly-Ala-Gln-Ala-Thr-Val-Asn-Gly-Ser-Gly-Met-Val-Leu-Ala-Asn-Pro-His-Phe-Pro-(Trp)-Gln -- - Ala-Glu-(Arg)-Phe-Tyr; and an 18-kilodalton subunit

having the amino-terminal sequence; His-Asp-Gly-Gly-Tyr-Ala-Ala-Leu-Ile-Arg-Arg-Ala-Ser-Tyr-Gly-Val-(Pro)-His-Ile-Thr-Ala-Asp-Asp-Phe; and which in the deacylation of echinocandin B as substrate exhibits optimal catalytic activity at about pH 6.0 at about 60° C in 0.05M KH_2PO_4 buffer:

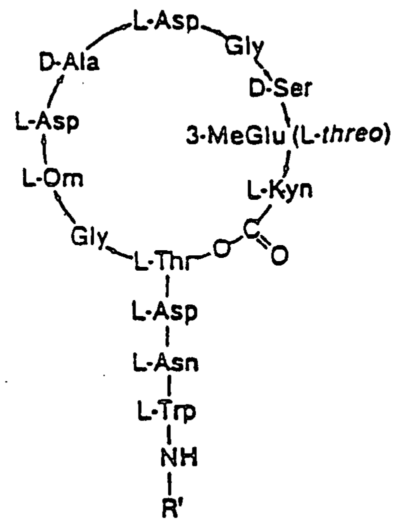
which comprises the steps, 1) heating for about one hour at a temperature of about 60° C an aqueous solution of crude deacylase buffered at about pH 6.0; 2) chromatographing the heat treated extract buffered at pH 6.0, 1.2M KCl and 14% $(NH_4)_2$ SO_4, over an hydrophobic interaction chromatogram; 3) fractionating by $(NH_4)_2SO_4$ the eluate containing 95% of deacylase activity from step 3 and separating the 10%

to 36% $(NH_4)_2SO_4$ fraction; 4) gel filtering said $(NH_4)_2SO_4$ fraction in pH 6.0 buffer containing 0.8M KCl and combining the fraction of the eluate containing 90% of the deacylas activity; 5) chromatographing said fractions in a pH 5.6, 0.05M KCl buffer on a cationexchange chromatogram and eluting said chromatogram with a linear gradient of KCl (0.05-0.5M) in said buffer; 6) adjusting the deacylase containing the eluate of step 5 to 0.05M KCl, chromatographing said eluate on a dye-ligand chromatogram in 0.05M KH_2PO_4, pH 6.0, and 0.05M KCl buffer and eluting said chromatogram with a linear gradient of KCl (0.05M-2.5M); 7) gel filtering the pooled eluate fractions from step 6 containing 80% of the deacylase activity in a 0.05M KH_2PO_4, pH 6.0, and 0.2M KCl buffer; and 8) adjusting to 0.04M KCl at pH 7 the deacylase containing eluate of step 7, chromatographing said eluate on a cation-exchange chromatogram in 0.05M KH_2PO_4, pH 7.0, and 0.04M KCl buffer and eluting the purified deacylase with a step-wise gradient of KCl (0.04-0.5-2M).

2. The process for deacylating a cyclicpeptide of the formula A or B



A



B

which comprises mixing said cyclicpeptide at a temperature between about 25°C and about 75°C in an aqueous medium at a pH between about 5 and about 7 with the purified deacylase prepared by the process of claim 1 where, in formula A, R is linoleoyl, myristoyl or palmitoyl and, in formula B, R[1] is decanoyl, 8-methyldecanoyl, 10-methyl-undecanoyl or 10-methyldodecanoyl; to provide the compound of the formula A or B wherein R or R[1] is hydrogen.

3. The process of claim 2 wherein the temperature is maintained at about 55° C to about 65° C.

4. The process of claim 2 wherein the aqueous medium contains an inorganic salt selected from an alkali metal chloride or an alkali metal nitrate.

5. The process of claim 4 wherein the salt is potassium chloride at a concentration of between about 0.1M to about 3.0M.

6. The process of claim 2 where, in formula A, R is linoleoyl.

7. The process of claim 2 where, in formula A, R is palmitoyl.

8. The process of claim 2 where, in formula B, R' is decanoyl.

9. The process of claim 2 wherein the aqueous medium contains dimethylsulfoxide at a concentration between about 5% and about 15%.

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(71) Applicant : ELI LILLY AND COMPANY
Lilly Corporate Center
Indianapolis Indiana 46285 (US)

(72) Inventor : Kreusman, Adam Joseph
3630 Redwood Drive
Indianapolis, Indiana 46227 (US)
Inventor : Yeh, Wu-Kuang
720 Primrose Court
Greenwood, Indiana 46142 (US)

(74) Representative : Tapping, Kenneth George et al
Erl Wood Manor
Windlesham Surrey, GU20 6PH (GB)

(54) **Lipopeptide deacylase.**

(57) Echinocandin B deacylase, a cell-associated enzyme from Actinoplanes utahensis, is purified to near homogeneity in a process comprising hydrophobic interaction chromatography, cation-exchange chromatography and gel filtrations. The enzyme is a heterodimer containing 18-KD and 63-KD subunits and is a simple enzyme unaffected in activity by co-factors, metal chelators, or sulfhydryl reagents. The enzyme catalyzes the deacylation of the lipid acyl portion of lipid cyclicpeptide metabolites such as ECB and aculeacin.

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Jouve, 18, rue Saint-Denis, 75001 PARIS

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Docket No. 342312003500

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 91 30 4976

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
D,A	JOURNAL OF BIOCHEMISTRY, vol. 105, no. 4, April 1989, TOKYO JP pages 606 - 610; HIDEO TAKESHIMA ET AL.: 'A Deacylation Enzyme for Aculeacin A, a Neutral Lipopeptide Antibiotic, from Actinoplanes utahensis: Purification and characterizarion' * the whole document *	1,2	C12N9/78 C07K7/08 C07K7/56 C12N9/80
D,A	ANNALS OF THE NEW YORK ACADEMY OF SCIENCES vol. 544, 1988, pages 152 - 167; M. DEBONO ET AL.: 'Syntehsis and Evaluation of LY121019, a Member of a Series of Semisynthetic Analogues of the Antifungal Lipopeptide Echinocandin B' * page 152, paragraph 1 -paragraph 2 * * page 153, paragraph 2 -paragraph 3 * * page 155, paragraph 4 - page 156, paragraph 4 *	3,7,8	
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)
A	EP-A-0 031 220 (ELI LILLY AND COMPANY) * page 1, line 1 - page 2, line 11 * * page 4, line 20 - page 5, line 8 * * page 10, line 10 - page 13, line 23 *	3,7,8	C12N C07K
D,A	JOURNAL OF ANTIBIOTICS, vol. 41, no. 8, 1988, TOKYO JP pages 1085 - 1091; LA VERNE D. BOECK ET AL.: 'Deacylation of A21978C, an acidic lipopeptide atibiotic complex, by Actinoplanes utahensis' * page 1085, paragraph 1 - page 1086, paragraph 2 * * page 1086, paragraph 5 *	1,3,9	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	19 FEBRUARY 1992	MONTERO LOPEZ B.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
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& : member of the same patent family, corresponding document

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(54) **Lipopeptide deacylase**

Lipopeptid Deacylase

Lipopeptide déacylase

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(73) Proprietor: ELI LILLY AND COMPANY
Indianapolis, Indiana 46285 (US)

(72) Inventors:
- Kreusman, Adam Joseph
  Indianapolis, Indiana 46227 (US)
- Yeh, Wu-Kuang
  Greenwood, Indiana 46142 (US)

(74) Representative: Tapping, Kenneth George et al
Lilly Industries Limited
European Patent Operations
Erl Wood Manor
Windlesham Surrey GU20 6PH (GB)

(56) References cited:
EP-A- 0 031 220

- J. of Biochemistry, vol.105, no. 4, April 1989, Tokyo JP, pp. 606-610; Hideo Takeshima et al.
- Annals of the New York Academy of Sciences, vol.544, 1988, pp. 152-167; M. Debond et al.
- Journal of Antibiotics, vol.41, no. 8, 1988, Tokyo JP, pp. 1085-1091; La Verne D. Boeck et al.

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Description

This invention relates to enzyme technology. In particular it relates to a lipopeptide deacylase in purified form produced by the organism Actinoplanes utahensis which deacylates the lipophilic acyl side chains of the antifungal metabolites echinocandin B (ECB), aculeacin, and analogs of ECB.

Echinocandin B and aculeacin are known cyclic hexapeptides having the linoleoyl and palmitoyl side chains respectively. Boeck, L. D., et al., 1988. J. Antibiot. (Tokyo), 41, 1085-1092; Boeck, L. D., et al., 1989 J. Antibiot (Tokyo), 42, 382-388; and Kimura, Y., et al., 1987. Agri. Biol. Chem. 51, 1617-1623; report the deacylation of the linoleoyl group of ECB with whole cells of Actinoplanes utahensis and Pseudomonas species. Takeshima, H., et al., 1989. J. Biochem. 105, 606-610, report the purification and partial characterization of a deacylase from A. utahensis which deacylates aculeacin.

Structural modification of the natural antifungals has led to potentially useful therapeutic agents such as cilofungin and daptomycin. Debono, M., et al., 1989. J. Antibiot. (Tokyo), 42, 389-397; Debono, M., et al., 1988. Ann. N.Y. Acad. Sci. 544, 152-167; Gordee, R.S. et al., 1988. Ann. N.Y. Acad. Sci. 544, 294-309; and Boeck, L.D., et al., 1988. J. Antibiot. (Tokyo), 41, 1085-1092. For example, ECB has been deacylated with whole cells of A. utahensis to cleave the lipophilic acyl side chain to provide the cyclic hexapeptide nucleus of ECB. Reacylation of the nucleus by chemical means has provided the acyl ECB compounds such as cilofungin with improved antifungal prperties.

Because of the need for improved antifungal agents for the treatment of systemic fungal infections methods for their production are important. Enzymatic methods for preparing such compounds are especially desirable since they are usually simpler and more economical methods than chemical methods. Accordingly the availability of enzymes useful for such conversions is highly desirable.

Actinoplanes utahensis deacylase is provided in purified form. The enzyme catalyzes the cleavage of the linoleoyl group of ECB and the palmitoyl group of aculeacin. The enzyme is a heterodimer comprising 63 KD and 18 KD subunits which is optimally active at pH 6.0 and 60° C. The enzyme is cell associated and is not affected by cofactors, metal ion chelators or sulfhydryl reagents.

The enzyme is useful in a method for the preparation of cyclic hexapeptide nuclei and in a method for the preparation of the cyclicpeptide nucleus of the A21978C antibiotics.

The invention also provides a method for purifying the deacylase enzyme to near homogeneity which comprises hydrophobic interaction, cation-exchange, dye-ligand chromatographies and gel filtrations.

The deacylase of Actinoplanes utahensis provided by this invention is referred to herein for convenience as ECB deacylase. The enzyme all of which is virtually cell-associated has the following physical, catalytic and kinetic properties in its purified state.

ECB deacylase is an 81-kilodalton (KD) heterodimer comprising 63-KD and 18 KD subunits. The amino-terminal sequences of the large and small subunits are respectively as follows.

Ser-Asn-Ala-Tyr-Gly-Leu-Gly-Ala-Gln-Ala-Thr-
Val-Asn-Gly-Ser-Gly-Met-Val-Leu-Ala-Asn-Pro-
His-Phe-Pro-(Trp)-Gln --- Ala-Glu-(Arg)-Phe-Tyr.

His-Asp-Gly-Gly-Tyr-Ala-Ala-Leu-Ile-Arg-Arg-Ala-
Ser-Tyr-Gly-Val-(Pro)-His-Ile-Thr-Ala-Asp-Asp-Phe.

In the above sequences the amino acid residues in parentheses indicate a tentative assignment while "---" indicates that the residue has not as yet been identified.

The amino acid composition of the purified enzyme is shown below in Table I. The composition was determined by the method described by Dotzlaf, J. E. and Yeh, W-K, 1987. J. Bacteriol. 169, 1611-1618.

TABLE I

Amino Acid Composition of A. utahensis Deacylase	
Amino Acid	Number of Residues per 81,000-dalton
Asp+Asn	74
Thr	51[a]
Ser	83[a]
Glu+Gln	45

[a] Determined by extrapolation to zero time of hydrolysis.

TABLE I (continued)

Amino Acid Composition of A. utahensis Deacylase	
Amino Acid	Number of Residues per 81,000-dalton
Pro	42
Gly	85
Ala	79
Cys	10[b]
Val	48
Met	5
Ile	25
Leu	53
Tyr	20
Phe	24
His	21
Lys	11
Arg	62
Trp	19[c]

[b] Determined by cysteic acid.

[c] Determined by hydrolysis in the presence of thioglycolic acid.

The molecular weight of ECB deacylase as estimated by gel filtration with Ultrogel AcA 44 was 46,000. The molecular weights of the subunits described above was determined by SDS-PAGE. The molecular weight determined by gel filtration is a significant underestimate that can be attributed to an abnormal gel elution behavior of the enzyme. The abnormal elution behavior of the enzyme on gel is typical of a membrane-bound protein attributable to the usually high hydrophobicity of the macromolecule.

ECB deacylase is a simple enzyme (although containing two subunits) that does not require any exogenous phospholipid, cofactor, metal ion or reducing agent for expression of its activity. Further, none of the common cofactors, metal ions or reducing agents stimulate the deacylase. Surprisingly, N-ethylmaleimide (NEM) enhances the rate of conversion of ECB to ECB nucleus about 6-7 fold and the purified deacylase.

An important property of the purified deacylase is its enhanced activity in the presence of high salt concentrations. The activity is increased by up to 3-fold in the presence of several common mono-and divalent metal salts. Salts such as sodium chloride, potassium chloride, magnesium chloride, calcium chloride, sodium or potassium nitrate are among such stimulatory salts. A preferred salt for this purpose is potassium chloride. Salt concentrations of about 0.1 molar up to about 3.0 molar appear to be the most favorable. Enhanced activity is observed at lower salt concentrations.

The lack of resolution observed for the deacylase by native-PAGE at pH 7 and 9 indicates that the isoelectric point of the enzyme is above 9. The native-PAGE was performed according to Blackshear, P. J. (1984) Methods Enzymol. 104, 237-255.

The substrate employed for study of the kinetic and catalytic properties of the purified enzyme was echinocandin B (ECB). ECB is essentially insoluble in aqueous solutions. Among several water miscible solvents tested for solubilization of ECB in aqueous media, dimethylsulfoxide (DMSO) at a low concentration of about 15% or lower was most compatible with the deacylase catalyzed reaction and subsequent enzyme activity analysis of HPLC.

The enzyme is optimally active at pH 6.0 at 60° C in 0.05M KH_2PO_4 buffer. It was found that the enzyme was at least twice as active when the reaction (ECB to ECB nucleus) was initiated with the enzyme rather than with the substrate.

The Km of the deacylase for echinocandin B, as determined by the Lineweaver-Burk method, was 50 µM.

The Vmax for the ECB reaction was 10-11 µmol of the peptide (ECB nucleus) formed/min/mg protein.

Regarding the reaction stoichiometry, the ratio of ECB nucleus formed to ECB disappearance was observed to be about 52.4%. The low ratio of conversion observed may be attributable to the occurrence of some other reaction or possibly to some degradation.

As was noted hereinabove the A. utahensis deacylase is cell-associated. For example, in a typical 90-hour culture broth of A. utahensis that exhibits a high total activity of ECB deacylase, over 99% of the deacylase activity was cell-associated. Less than 5% of the cell-associated deacylase activity was released by incubation of the cells in 0.01M KH_2PO_4, pH 6.0 for one day. Increase of the ionic strength of this and other buffers had only a slight effect in recovery of a soluble deacylase. However it has been found that by treating the cells with salts such as potassium or sodium chloride, potassium or sodium nitrate and magnesium or calcium salts, a high recovery (60 to 80%) of soluble deacylase

is realized. The effect of this salt-treatment is shown in Table II below.

Table II

Solubilization of ECB Deacylase From A. Utahensis		
Cell Treatment[a]	Activity Distribution (%)	Specific Activity (U/mg protein)
KCl/KH_2PO_4 and Sonication		
Supernatant Fraction	80	0.1
Pellet Fraction	20	-
KCl/KH_2PO_4 Only		
Supernatant Fraction	60-80	0.2-0.4
Pellet Fraction	20-40	-
KH_2PO_4 and Sonication		
Supernatant Fraction	80	0.1
Pellet Fraction	20	-
KH_2PO_4 Only		
Supernatant Fraction	0	-
Pellet Fraction	100	-

[a] A. utahensis cells were resuspended in 0.8 M $KCl/0.05M\ KH_2PO_4$ or 0.05M KH_2PO_4 only, pH 6.0, sonicated continually for one minute, wherever specified, and centrifuted at 48,000 x g for one hour.

The ECB deacylase is produced by culturing Actinoplanes utahensis under submerged aerobic fermentation conditions. The fermentation method and the conditions employed are known, Boeck, L. D., Fukuda, D. S., Abbott, B. J. and Debono, M., 1989. J. Antibiot. (Tokyo), 42, 382-388. Maximum production of the deacylase activity occurs at about 90 hours after inoculation of the culture medium. As described above and in Example 1 hereinafter the enzyme in crude form is solubilized by salt treatment of the whole cells preferably with potassium chloride.

The crude solubilized enzyme is purified to near homogenity in the purification process of the invention which comprises hydrophobic interaction chromatography, cation-exchange chromatography and gel filtration steps. As described above herein the ECB deacylase behaves as a loosely cell-bound protein and is solubilized differentially by treating whole cells of A. utahensis with an inorganic salt. Preferably potassium chloride is used. The solubilized enzyme solution is desirably filtered and the filtrate concentrated by ultrafiltration to provide a more concentrated deacylase solution for the ensuing steps.

In the first step of the process the concentrated cell extract is heated for one hour at about 60° C and is treated while warm with ammonium sulfate, 14%, and potassium chloride, 1.2M. The heat treatment causes other proteins in the solution which are unstable at 60° C to precipitate. The ECB deacylase is stable at 60° C and remains solubilized.

The heat treated extract is then subjected to hydrophobic interaction chromatography at a temperature of about 25° C on a hydrophobic interaction chromatographic material such as lipid substituted agarose for example the commercially available Octyl Sepharose (Pharmacia). The column is equilibrated to pH 6 with about 0.05M potassium dihydrogen phosphate or other suitable buffer, 1.2M potassium chloride and 14% ammonium sulfate. The column is desirably washed with the same buffer and the bound protein is eluted with a simultaneous linear gradient of KCl (1.2-0.1M) and $(NH_4)_2SO_4$ (14-0%) in 0.05M KH_2PO_4, pH 6 buffer. The ECB deacylase is eluted as a single, unsymmetrical activity peak.

The peak fractions containing about 95% of the total deacylase activity are pooled and subjected to ammonium sulfate fractionation. The 10-36% $(NH_4)_2SO_4$ fraction is collected and subjected to gel filtration in pH 6 buffer containing 0.8M KCl. Gel types which can be used may vary however Sephacryl S-200 HR is a suitable gel. The enzyme is eluted as one main activity peak and a minor activity peak. The peak fractions which contain 90% of the deacylase activity from the main peak are pooled and adjusted to 0.05 M KCl at pH 5.6. The pooled fractions are subjected to cation-exchange chromatography over a suitable cation-exchange resin such as one of the commercially available Trisacryl resins for example, Trisacryl-CM (IBF Biotechnics). The column is equilibrated with 0.05M KH_2PO_4, pH 5.6, and 0.05M KCl. The column is washed with the same buffer and the bound proteins are eluted with a linear gradient of KCl (0.05M - 0.5M) in the same buffer. The deacylase enzyme is eluted as two activity peaks.

The fractions containing the deacylase activity from each of the peaks are pooled, adjusted to 0.05M KCl, and applied to a dye-ligand gel such as Red-Sepharose (Pharmacia, Inc.) or Dyematrex Blue A (Amicon). The gel is equil-

ibrated prior to use with 0.05M KH_2PO_4, pH 6.0, and 0.05M KCl. The bound proteins are eluted preferably with a step-wise gradient of KCl (0.05-2-3.3M) in the same buffer from the Red-Sepharose or preferably with a linear gradient of KCl (0.05 - 2.5M) in the same buffer from the Blue A gel. A broad and unsymmetrical activity peak is obtained from the dye-ligand chromatography.

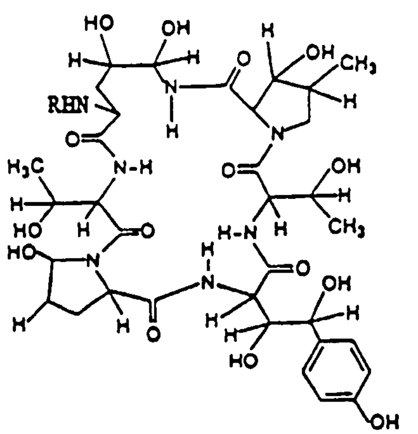
The peak fractions containing about 80% of the total activity from the dye-ligand gel are pooled and subjected to gel filtration over a gel of the type such as Ultragel AcA 44 (IBF Biotechnics) previously equilibrated with 0.05M KH_2PO_4, pH 6.0, and 0.2M KCl. The enzyme is eluted as a single activity peak and the peak fractions containing all of the deacylase are pooled.

The pooled fractions are adjusted to 0.04M KCl at pH 7.0 and then again subjected to cation exchange chromatography over a negatively charged resin such as Trisacryl-CM. The resin is equilibrated prior to use with 0.05M KH_2PO_4, pH 7.0, and 0.04M KCl buffer and bound deacylase is eluted with a step-wise gradient of KCl (0.04 - 0.5 - 2M) in the same buffer. One broad activity peak and one sharp minor activity peak are observed. The fractions from these two activity peaks of the purified enzyme can be stored at -70° C for further use.

During the foregoing 8-step process for the purification of ECB deacylase two size-forms and two charge-forms of the enzyme are observed. The major peak obtained with the last cation-exchange chromatography was analyzed by SDS-PAGE and showed two closely slow-moving bands and two closely fast-moving bands. The minor cation-exchange chromatography peak showed a single slow-moving band and two fast-moving bands. The absence of the second slow-moving band suggests that this protein is likely a degradation product from the first slow-moving band.

The purified ECB deacylase provided by the purification process of the invention is useful in a method for deacylating lipo cyclicpeptides to provide the cyclicpeptide nuclei thereof. In particular the enzyme deacylates the cyclohexapeptides echinocandin B and aculeacin. Further the purified enzyme cleaves the fatty acid side chain from the cyclicpeptide A21978 antibiotic factors including Daptomycin.

The process of this invention comprises mixing at a temperature between about 25° C and 75° C in an aqueous medium at a pH between about 5 and about 7 a cyclicpeptide represented by the formula A or B



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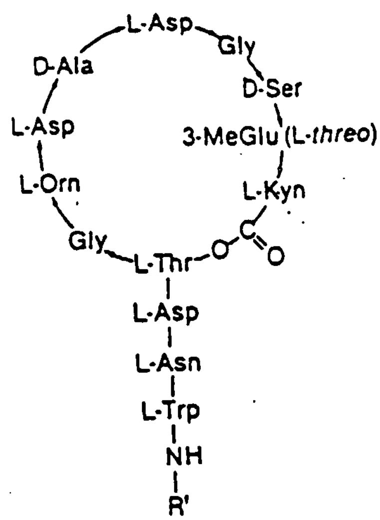


B

with echinocandin B deacylase where, in formula A, R is linoleoyl, myristoyl or palmitoyl and, in formula B, R' is decanoyl, 8-methyldecanoyl, 10-methylundecanoyl, or 10-methyldodecanoyl; to provide the compound of the formula A or B wherein R and R' are hydrogen.

The process is preferably carried out at a temperature between about 55° C and about 65° C. The pH of the medium can be maintained with a suitable buffer. A salt such as an alkali metal chloride eg. KCl or NaCl or an alkali metal nitrate eg, KNO3 appears to have a beneficial effect on the activity of the enzyme and can be incorporated in the aqueous medium. Preferably the salt is KCl at a concentration of about 0.05M to about 3.0M.

A water miscible solvent also can be added to the medium in the instance where the substrate's water solubility is low. For example, echinocandin B has a low solubility in water. Dimethylsulfoxide (DMSO) can be added to the reaction medium to enhance its solubility. DMSO is also compatible with the deacylase. In general DMSO can be added in amounts between about 5% and 15% v:v. as demonstrated in the case of ECB and aculeacin.

The process is preferably carried out by forming a solution of the substrate in the buffered aqueous medium,

warming the mixture to the reaction temperature and adding the enzyme. The reaction mixture is stirred, shaken or otherwise agitated to provide good contact of substrate and enzyme. The reaction mixture can be monitored from time to time by assaying small aliquots of the mixture by the assay method described hereinafter. Alternatively, the solution of the substrate can be added to the buffered enzyme solution. However, better deacylation results are generally obtained by adding the enzyme to the substrate.

If, as determined by monitoring the reaction, the reaction is proceeding too slowly or has terminated prematurely additional fresh enzyme can be added to complete the deacylation.

The process also may be carried out with an immobilized form of the enzyme. The enzyme may be bonded to a suitable inert resin support and packed into a column. The aqueous buffered solution can be applied to the column and washed through with buffer. Recycling may be required to achieve complete conversion.

A preferred embodiment of the process of the invention comprises the deacylation of echinocandin B (formula A, R = linoleoyl) to the echinocandin B nucleus (formula A, R = H). Another preferred embodiment comprises the deacylation of aculeacin (formula A, R = palmitoyl) to the same ECB nucleus.

The substrate specificity studies carried out with the purified deacylase of the invention revealed rather broad specificity for the cyclicpeptides of the echinocandin B type formula A and the A21978 antibiotic type of formula B. The A21978 substrates are known metabolites described by U.S. Patent No. 4,537,717. Also found to be substrates for the deacylase are certain derivatives of the ECB nucleus which are prepared by the acylation of the nucleus. Examples of such acyl derivatives are represented by the above formula A when R is a 3-phenylpropionyl group substituted in the para position by $C_7H_{15}O$-(30%), $C_5H_{11}O$-(12%); a phenylacetyl group substituted in the para position by C_8H_7O-(30%) or a $C_{11}H_{23}C(O)NH$-group (5%); a benzoyl group substituted in the para position by a $C_{11}H_{23}C(O)NH$-group; or a cinnamoyl group substituted in the para position by a $C_{11}H_{23}C(O)NH$-group (15%). The figures in parentheses are the percent activity relative to the deacylase activity of 100% for ECB itself.

The following example further illustrates and describes the invention but is not intended to be limiting thereof.

Example 1

Preparation and Purification of ECB Deacylase

A. Fermentation of Actinoplanes utahensis Actinoplanes utahensis NRRL 12052 was grown in a 150-liter fermenter under the conditions described by Boeck, L. D., Fukuda, D. S., Abbott, B. J., and Debono, M. 1989. J. Antibiot. (Tokyo), 42, 382-388. Cells containing a high activity of echinocandin B deacylase (90 hours after inoculation) were harvested by centrifugation; washed with 0.05M KH_2PO_4, pH 6.0, and used for enzyme isolation and purification.

B. Enzyme Solubilization and Purification Unless otherwise specified the following isolation and purification procedures were carried out at a temperature between about 0° C and 4° C.

Assay of 100 liters of fermentation medium at 90 hours showed that virtually all of the deacylase activity was cell-associated. The assay method employed throughout this example is described hereinafter.

Fresh cells (7.9 kg wet weight) were re-suspended in 0.05M KH_2PO_4, pH 6.0, with 0.8M KCl to a total volume of 57 liters and the suspension was stirred continuously for one day. Most of the cell-associated deacylase activity became soluble differentially by this salt treatment.

The solubilized deacylase was filtered through Whatman No. 1 paper and the filtrate was concentrated to a volume of 3.3 liters with an Amicon YM 30 spiral ultrafiltration cartridge. The concentrated extract was heated at 60°C for one hour.

The heat-treated enzyme extract was treated with $(NH_4)_2SO_4$ at 14% concentration and KCl at 1.2M and was loaded onto a Octyl-Sepharose column (5 x 36 cm) previously equilibrated with 0.05 M KH_2PO_4, pH 6.0, 1.2M KCl and 14% $(NH_4)_2SO_4$. The column was washed with two bed volumes of the same buffer and bound proteins were eluted with a simultaneous linear gradient of KCl (1.2-0.1M) and $(NH_4)_2SO_4$ (14-0%) in 0.05M KH_2PO_4, pH 6.0. This chromatography (hydrophobic interaction chromatography) was carried out at a temperature of about 25° C. ECB deacylase was eluted as a single but non-symmetrical activity peak.

The peak fractions containing 95% of the total deacylase activity were pooled and fractionated with $(NH_4)_2SO_4$. The 10-36% $(NH_4)_2SO_4$ fraction was loaded onto a Sephacryl S-200 HR column (5 x 69 cm) previously equilibrated with 0.05M KH_2PO_4, pH 6.0, and 0.8M KCl (buffer A). The deacylase was eluted as a main activity peak and a minor one. The peak fractions containing 90% of the enzyme activity from the main peak were pooled, adjusted to 0.05M KCl at pH 5.6 and were applied to a Triascryl-CM column (2.5 x 33 cm) previously equilibrated with 0.05M KH_2PO_4, pH 5.6, and 0.05M KCl (buffer B). The column was washed with two-bed volumes of buffer B and bound proteins were eluted with a linear gradient of KCl (0.05-0.5M) in buffer B. The deacylase was eluted as two activity peaks.

The fractions containing the deacylase activity from each of the two peaks were pooled, one pool per peak. The two enzyme pools were adjusted to 0.05M KCl and one pool loaded onto a Red-Sepharose column (3.2 x 15.5 cm) and the other onto a Dyematrex Blue A column (2.2 x 22 cm). Both columns were previously equilibrated with 0.05M KH_2PO_4, pH 6.0, and 0.05M KCl (buffer C). After both columns were washed with two bed volumes of buffer C the bound protein was eluted from the Red-Sepharose column with a step-wise gradient of KCl (0.05-2-3.3 M) in buffer C and from the Blue A column with a linear gradient of KCl (0.05-2.5 M) in buffer C. A broad and non-symmetrical activity peak was observed from each dye-ligand chromatography.

The peak fractions containing 80% of the total deacylase activity from the Red-Sepharose peak were pooled and applied to a Ultragel Ac 44 column (1 x 118 cm) previously equilibrated with 0.05 M KH_2PO_4, pH 6.0, and 0.2M KCl (buffer D). The deacylase was eluted as a single activity peak.

The peak fractions containing all of the deacylase activity were pooled, adjusted to 0.04M KCl at pH 7.0 and loaded onto a Trisacryl-CM column (1 x 34 cm) previously equilibrated with 0.05M KH_2PO_4, pH 7.0 and 0.04M KCl (buffer E). After the column was washed with two bed volumes of buffer E bound proteins were eluted with a step-wise gradient of KCl (0.04-0.5-2M) in buffer E. One broad main activity peak and one sharp minor activity peak were observed. The fractions from the two activity peaks were stored individually at -70° C until required.

The following Table III shows the results obtained with each step of the isolation and purification of ECB deacylase detailed above.

Table III

Purification of ECB Deacylase

Step	Protein (mg)	Activity (U)	Sp. Act. (U/mg)	Recovery (%)
Soluble Extract	12,546	4,631	0.37	100
Heat-Treated Extract (60° C for 1-hr)	7,325	3,606	0.49	78
Octyl-Sepharose Eluate	1,357	2,038	1.5	44
10-36% $(NH_4)_2SO_4$ Fraction	1,077	1,513	1.41	33
Sephacryl S-200 HR Eluate	857	1,470	1.72	32
Trisacryl-CM Eluate, pH 5.6 (A)	78	293	3.76	6.3
Trisacryl-CM Eluate, pH 5.6 (B)	175	595	3.42	12.8
Red-Sepharose Eluate (A1)	10.1	58.4	5.78	1.3
Blue A Eluate (B1)	113	368	3.53	7.9
Ultrogel AcA 44 Eluate (Ala)	8.1	60.2	7.42	1.3
Trisacryl-CM Eluate, pH 7.0 (Ala1)	5.5	56.2	10.22	1.2
Trisacryl-CM Eluate, pH 7.0 (Ala2)	1.2	13.5	11.25	0.3

As described hereinabove, two activity peaks were observed in the first and second cation-exchange chromatography with Trisacryl-CM. The fractions from each of the two peaks A and B of the first chromatography were collected and analyzed separately as shown. The pooled A fractions were subjected to the Red-Sepharose dye-ligand chromatography (A1) while those of the second activity peak B were subjected to the Dyematrex Blue A dye-ligand chromatography (B1). When the eluates of each dye-ligand chromatography were analyzed the specific activity increased for the Red-Sepharose treatment while the specific activity for the Blue A increased only slightly. Accordingly, in this in-

stance, the eluate of the Red-Sepharose column was selected for further purification by Ultrogel filtration (A1a) followed by cation-exchange chromatography with Trisacryl-CM. Again, as with the previous cation-exchange chromatography the two activity peaks (enzyme forms) were collected and analyzed separately.

Enzyme assay

The assay employed herein for determining and measuring deacylase activity utilizes echinocandin B as substrate. A typical reaction mixture of 1 ml for ECB deacylase assay contained 425 µmole of ECB and 0.000003 to 0.003 unit of the enzyme in 0.05M KH_2PO_4, pH 6.0, in the presence of 0.68M KCl and 15% DMSO to effect solution of the ECB. The enzymatic reaction was initiated by addition of the enzyme and was continued for 20 min at 60° C before being interrupted by the addition of phosphoric acid. After a low-speed centrifugation to remove precipitated protein, the deacylase activity was determined by monitoring the formation of ECB nucleus at 225 nm using HPLC.

HPLC components were IBM PS/2 Model 80 and Color Display (IBM, Armonk, N.Y.), a Water 715 Ultra WISP Sample Processor (Waters Associates, Milford, MA), and a Beckman System Gold Programmable Solvent Module 126 and Scanning Detector Module 167 (Beckman, Fullerton, CA.).

The ECB nucleus was eluted from an Apex Octadecyl 3 µ column (4.6 x 10 cm) (Jones Chromatography, Littleton, Co.) with a mobile phase of 3.9% CH_3CN/0.1% trifluoroacetic acid and a flow rate of 1 ml/min. The nucleus formation was linear with time during the assays. Duplicated HPLC analyses had an average 2-3% deviation. As used herein one unit of enzyme activity is defined as the amount of the deacylase required to cause formation of one µmole of the ECB nucleus per minute from ECB under the above described reaction conditions.

The specific activity (Table III) is defined as units per mg of protein. The protein content was determined by the method of Bradford using bovine serum albumin as standard (Bradford, M. M., (1976) Anal. Biochem. 72, 248-254.

Claims

Claims for the following Contracting States : AT, BE, CH, DE, DK, FR, GB, GR, IT, LI, NL, SE

1. Echinocandin B deacylase in purified form which is an 81-kilodalton heterodimer having the following amino acid composition:

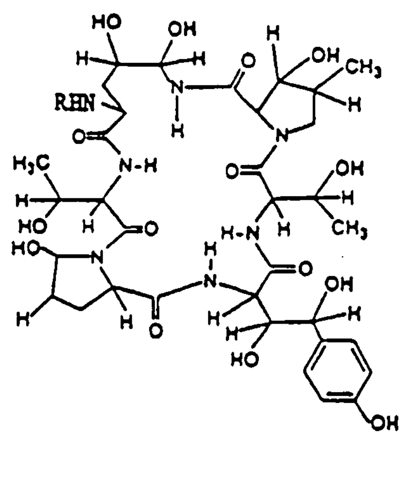
Amino Acid	Number of Residues per 81,000-dalton
Asp+Asn	74
Thr	51
Ser	83
Glu+Gln	45
Pro	42
Gly	85
Ala	79
Cys	10
Val	48
Met	5
Ile	25
Leu	53
Tyr	20
Phe	24
His	21
Lys	11
Arg	62
Trp	19

which has a specific activity of between about 10 U/mg and about 11.25 U/mg; which has a 63-kilodalton subunit having the amino-terminal sequence:
Ser-Asn-Ala-Tyr-Gly-Leu-Gly-Ala-Gln-Ala-Thr-Val-Asn-Gly-Ser-Gly-Met-Val-Leu-Ala-Asn-Pro-His-Phe-Pro-(Trp)-

Gln --- Ala-Glu-(Arg)-Phe-Tyr; and an 18-kilodalton subunit having the amino-terminal sequence; His-Asp-Gly-Gly-Tyr-Ala-Ala-Leu-Ile-Arg-Arg-Ala-Ser-Tyr-Gly-Val-(Pro)-His-Ile-Thr-Ala-Asp-Asp-Phe; and which in the deacylation of echinocandin B as substrate exhibits optimal catalytic activity at about pH 6.0 at about 60° C in 0.05M KH_2PO_4 buffer.

2. The process for preparing the purified deacylase of claim 1 which comprises the steps, 1) heating for about one hour at a temperature of about 60° C an aqueous solution of crude deacylase buffered at about pH 6.0; 2) chromatographing the heat treated extract buffered at pH 6.0, 1.2M KCl and 14% $(NH_4)_2SO_4$, over an hydrophobic interaction chromatogram; 3) fractionating by $(NH_4)_2SO_4$ the eluate containing 95% of deacylase activity from step 3 and separating the 10% to 36% $(NH_4)_2SO_4$ fraction; 4) gel filtering said $(NH_4)_2SO_4$ fraction in pH 6.0 buffer containing 0.8M KCl and combining the fraction of the eluate containing 90% of the deacylase activity; 5) chromatographing said fractions in a pH 5.6, 0.05M KCl buffer on a cation-exchange chromatogram and eluting said chromatogram with a linear gradient of KCl (0.05-0.5M) in said buffer; 6) adjusting the deacylase containing the eluate of step 5 to 0.05M KCl, chromatographing said eluate on a dye-ligand chromatogram in 0.05M KH_2PO_4, pH 6.0, and 0.05M KCl buffer and eluting said chromatogram with a linear gradient of KCl (0.05M-2.5M); 7) gel filtering the pooled eluate fractions from step 6 containing 80% of the deacylase activity in a 0.05M KH_2PO_4, pH 6.0, and 0.2M KCl buffer; and 8) adjusting to 0.04M KCl at pH 7 the deacylase containing eluate of step 7, chromatographing said eluate on a cation-exchange chromatogram in 0.05M KH_2PO_4, pH 7.0, and 0.04M KCl buffer and eluting the purified deacylase with a step-wise gradient of KCl (0.04-0.5-2M).

3. The process for deacylating a cyclicpeptide of the formula A or B



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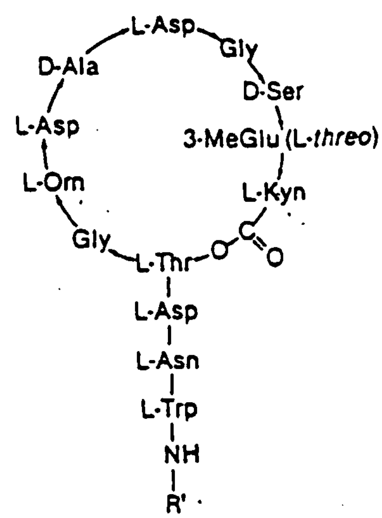

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which comprises mixing said cyclicpeptide at a temperature between about 25° C and about 75° C in an aqueous medium at a pH between about 5 and about 7 with the purified deacylase of claim 1 where, in formula A, R is linoleoyl, myristoyl or palmitoyl and, in formula B, R' is decanoyl, 8-methyldecanoyl, 10-methyl-undecanoyl or 10-methyldodecanoyl; to provide the compound of the formula A or B wherein R or R' is hydrogen.

4. The process of claim 3 wherein the temperature is maintained at about 55° C to about 65° C.

5. The process of claim 3 wherein the aqueous medium contains an inorganic salt selected from an alkali metal chloride or an alkali metal nitrate.

6. The process of claim 5 wherein the salt is potassium chloride at a concentration of between about 0.1M to about 3.0M.

7. The process of claim 3 where, in formula A, R is linoleoyl.

8. The process of claim 3 where, in formula A, R is palmitoyl.

9. The process of claim 3 where, in formula B, R' is decanoyl.

10. The process of claim 3 wherein the aqueous medium contains dimethylsulfoxide at a concentration between about 5% and about 15%.

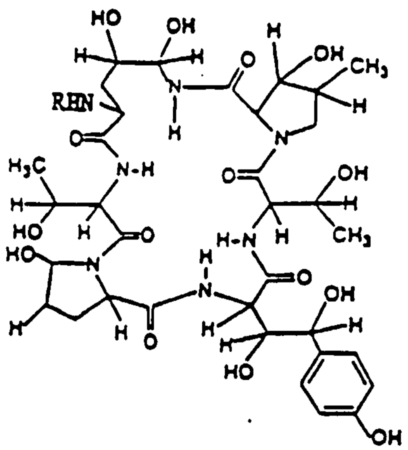
Claims for the following Contracting State : ES

1. The process for preparing Echinocandin B deacylase in purified form which is an 81-kilodalton heterodimer having the following amino acid composition:

Amino Acid	Number of Residues per 81,000-dalton
Asp+Asn	74
Thr	51
Ser	83
Glu+Gln	45
Pro	42
Gly	85
Ala	79
Cys	10
Val	48
Met	5
Ile	25
Leu	53
Tyr	20
Phe	24
His	21
Lys	11
Arg	62
Trp	19

which has a specific activity of between about 10 U/mg and about 11.25 U/mg; which has a 63-kilodalton subunit having the amino-terminal sequence:
Ser-Asn-Ala-Tyr-Gly-Leu-Gly-Ala-Gln-Ala-Thr-Val-Asn-Gly-Ser-Gly-Met-Val-Leu-Ala-Asn-Pro-His-Phe-Pro-(Trp)-Gln--- Ala-Glu-(Arg)-Phe-Tyr; and an 18-kilodalton subunit having the amino-terminal sequence; His-Asp-Gly-Gly-Tyr-Ala-Ala-Leu-Ile-Arg-Arg-Ala-Ser-Tyr-Gly-Val-(Pro)-His-Ile-Thr-Ala-Asp-Asp-Phe; and which in the deacylation of echinocandin B as substrate exhibits optimal catalytic activity at about pH 6.0 at about 60° C in 0.05M KH_2PO_4 buffer:
which comprises the steps, 1) heating for about one hour at a temperature of about 60° C an aqueous solution of crude deacylase buffered at about pH 6.0; 2) chromatographing the heat treated extract buffered at pH 6.0, 1.2M KCl and 14% $(NH_4)_2SO_4$, over an hydrophobic interaction chromatogram; 3) fractionating by $(NH_4)_2SO_4$ the eluate containing 95% of deacylase activity from step 3 and separating the 10% to 36% $(NH_4)_2SO_4$ fraction; 4) gel filtering said $(NH_4)_2SO_4$ fraction in pH 6.0 buffer containing 0.8M KCl and combining the fraction of the eluate containing 90% of the deacylase activity; 5) chromatographing said fractions in a pH 5.6, 0.05M KCl buffer on a cationexchange chromatogram and eluting said chromatogram with a linear gradient of KCl (0.05-0.5M) in said buffer; 6) adjusting the deacylase containing the eluate of step 5 to 0.05M KCl, chromatographing said eluate on a dye-ligand chromatogram in 0.05M KH_2PO_4, pH 6.0, and 0.05M KCl buffer and eluting said chromatogram with a linear gradient of KCl (0.05M-2.5M); 7) gel filtering the pooled eluate fractions from step 6 containing 80% of the deacylase activity in a 0.05M KH_2PO_4, pH 6.0, and 0.2M KCl buffer; and 8) adjusting to 0.04M KCl at pH 7 the deacylase containing eluate of step 7, chromatographing said eluate on a cation-exchange chromatogram in 0.05M KH_2PO_4, pH 7.0, and 0.04M KCl buffer and eluting the purified deacylase with a step-wise gradient of KCl (0.04-0.5-2M).

2. The process for deacylating a cyclicpeptide of the formula A or B



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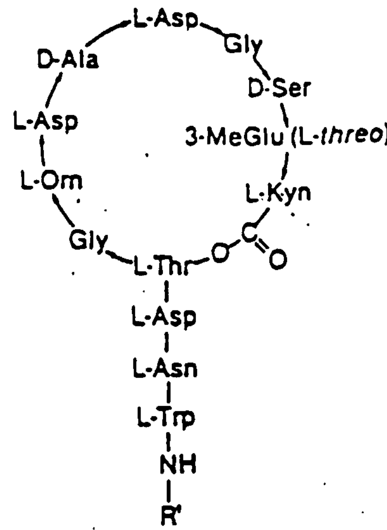

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which comprises mixing said cyclicpeptide at a temperature between about 25°C and about 75°C in an aqueous medium at a pH between about 5 and about 7 with the purified deacylase prepared by the process of claim 1 where, in formula A, R is linoleoyl, myristoyl or palmitoyl and, in formula B, R[1] is decanoyl, 8-methyldecanoyl, 10-methyl-undecanoyl or 10-methyldodecanoyl; to provide the compound of the formula A or B wherein R or R[1] is hydrogen.

3. The process of claim 2 wherein the temperature is maintained at about 55° C to about 65° C.

4. The process of claim 2 wherein the aqueous medium contains an inorganic salt selected from an alkali metal chloride or an alkali metal nitrate.

5. The process of claim 4 wherein the salt is potassium chloride at a concentration of between about 0.1M to about 3.0M.

6. The process of claim 2 where, in formula A, R is linoleoyl.

7. The process of claim 2 where, in formula A, R is palmitoyl.

8. The process of claim 2 where, in formula B, R' is decanoyl.

9. The process of claim 2 wherein the aqueous medium contains dimethylsulfoxide at a concentration between about 5% and about 15%.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten : AT, BE, CH, DE, DK, FR, GB, GR, IT, LI, NL, SE

1. Echinocandin-B-Deacylase in gereinigter Form, die ein 81 Kilodalton Heterodimer mit der folgenden Aminosäurezusammensetzung ist

Aminosäure	Anzahl der Reste pro 81000 Dalton
Asp+Asn	74
Thr	51

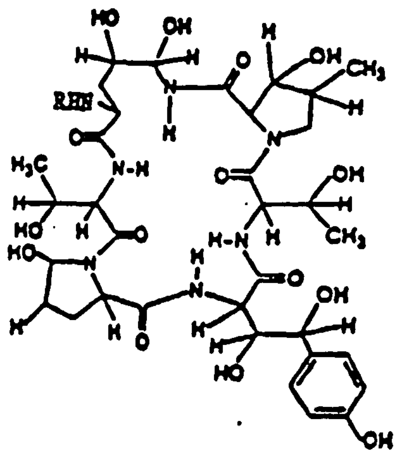
(fortgesetzt)

Aminosäure	Anzahl der Reste pro 81000 Dalton
Ser	83
Glu+Gln	45
Pro	42
Gly	85
Ala	79
Cys	10
Val	48
Met	5
Ile	25
Leu	53
Tyr	20
Phe	24
His	21
Lys	11
Arg	62
Trp	19

die eine spezifische Aktivität zwischen etwa 10 E/mg und etwa 11,25 E/mg aufweist, die eine Untereinheit mit 63 Kilodalton mit der folgenden aminoterminalen Sequenz aufweist: Ser-Asn-Ala-Tyr-Gly-Leu-Gly-Ala-Gln-Ala-Thr-Val-Asn-Gly-Ser-Gly-Met-Val-Leu-Ala-Asn-Pro-His-Phe-Pro-(Trp)-Gln --- Ala-Glu-(Arg)-Phe-Tyr und eine Untereinheit mit 18 Kilodalton mit der folgenden aminoterminalen Sequenz aufweist: His-Asp-Gly-Gly-Tyr-Ala-Ala-Leu-Ile-Arg-Arg-Ala-Ser-Tyr-Gly-Val-(Pro)-His-Ile-Thr-Ala-Asp-Asp-Phe und die bei der Deacylierung von Echinocandin B als Substrat eine optimale katalytische Aktivität bei etwa pH 6,0 bei etwa 60°C in 0,05 M KH_2PO_4 Puffer aufweist.

2. Verfahren zur Herstellung der gereinigten Deacylase nach Anspruch 1, gekennzeichnet durch folgende Schritte 1) Erhitzen einer bei etwa pH 6,0 gepufferten wäßrigen Lösung der rohen Deacylase für etwa eine Stunde bei einer Temperatur von etwa 60°C, 2) Chromatographie des bei pH 6,0, 1,2 M KCl und 14 % $(NH_4)_2SO_4$ gepufferten hitzebehandelten Extrakts über ein hydrophobes Interaktionschromatographiematerial, 3) Fraktionierung des 95 % der Deacylaseaktivität enthaltenden Eluats von Schritt 2 durch $(NH_4)_2SO_4$ und Abtrennung der 10 % bis 36 % $(NH_4)_2SO_4$ Fraktion, 4) Gelfiltration dieser $(NH_4)_2SO_4$ Fraktion in 0,8 M KCl enthaltendem Puffer mit pH 6,0 und Vereinigen der Eluatsfraktion, die 90 % der Deacylaseaktivität enthält, 5) Chromatographie dieser Fraktionen in einem 0,05 M KCl Puffer mit pH 5,6 auf einem Kationenaustauchchromatographiematerial und Elution dieses Chromatographiematerials mit einem linearen KCl Gradienten (0,05-0,5 M) in diesem Puffer, 6) Einstellen des die Deacylase enthaltenden Eluats von Schritt 5 auf 0,05 M KCl, Chromatographie dieses Eluats auf einem Farbstoffligandenchromatographiematerial in einem Puffer aus 0,05 M KH_2PO_4, und 0,05 M KCl mit pH 6,0 und Elution dieses Chromatographiematerials mit einem linearen KCl Gradienten (0,05 M-2,5 M), 7) Gelfiltration der vereinigten Eluatfraktionen von Schritt 6, die 80 % der Deacylaseaktivität enthalten in einem Puffer aus 0,05 M KH_2PO_4 und 0,2 M KCl mit pH 6,0 und 8) Einstellen des die Deacylase enthaltenden Eluats von Schritt 7 auf 0,04 M KCl bei pH 7, Chromatographie dieses Eluats auf einem Kationenaustauscherchromatographiematerial in einem Puffer aus 0,05 M KH_2PO_4 und 0,04 M KCl mit pH 7,0 und Elution der gereinigten Deacylase mit einem stufenweisen KCl Gradienten (0,04-0,5-2 M).

3. Verfahren zur Deacylierung eines Cyclopeptids der Formel A oder B



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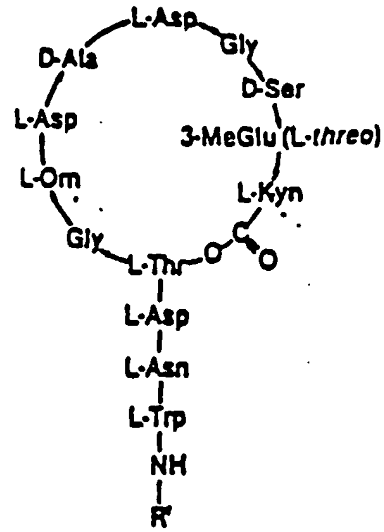

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gekennzeichnet durch Mischen dieses Cyclopeptids bei einer Temperatur zwischen etwa 25°C und etwa 75°C in einem wäßrigen Medium bei einem pH zwischen etwa 5 und etwa 7 mit der gereinigten Deacylase nach Anspruch 1, worin in Formel A R für Linoleoyl, Myristoyl oder Palmitoyl steht und in Formel B R' für Decanoyl, 8-Methyldecanoyl, 10-Methylundecanoyl oder 10-Methyldodecanoyl steht, unter Bereitstellung der Verbindung der Formel A oder B, worin R oder R' für Wasserstoff steht.

4. Verfahren nach Anspruch 3, worin die Temperatur bei etwa 55°C bis etwa 65°C gehalten wird.

5. Verfahren nach Anspruch 3, worin das wäßrige Medium ein anorganisches Salz enthält, das aus einem Alkalimetallchlorid oder einem Alkalimetallnitrat ausgewählt ist.

6. Verfahren nach Anspruch 5, worin das Salz Kaliumchlorid in einer Konzentration zwischen etwa 0,1 M bis etwa 3,0 M ist.

7. Verfahren nach Anspruch 3, worin in Formel A R für Linoleoyl steht.

8. Verfahren nach Anspruch 3, worin in Formel A R für Palmitoyl steht.

9. Verfahren nach Anspruch 3, worin in Formel B R' für Decanoyl steht.

10. Verfahren nach Anspruch 3, worin das wäßrige Medium Dimethylsulfoxid in einer Konzentration zwischen etwa 5 % und etwa 15 % enthält.

Patentansprüche für folgenden Vertragsstaat : ES

1. Verfahren zur Herstellung von Echinocandin-B-Deacylase in gereinigter Form, die ein 81 Kilodalton Heterodimer mit der folgenden Aminosäurezusammensetzung ist

Aminosäure	Anzahl der Reste pro 81000 Dalton
Asp+Asn	74
Thr	51
Ser	83
Glu+Gln	45

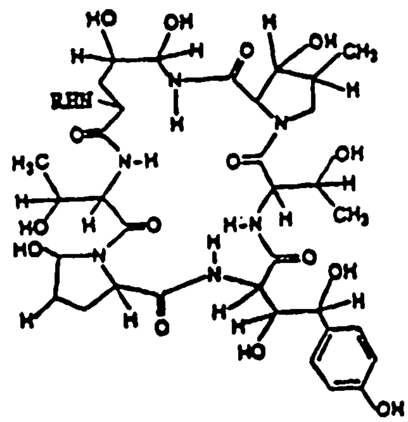
(fortgesetzt)

Aminosäure	Anzahl der Reste pro 81000 Dalton
Pro	42
Gly	85
Ala	79
Cys	10
Val	48
Met	5
Ile	25
Leu	53
Tyr	20
Phe	24
His	21
Lys	11
Arg	62
Trp	19

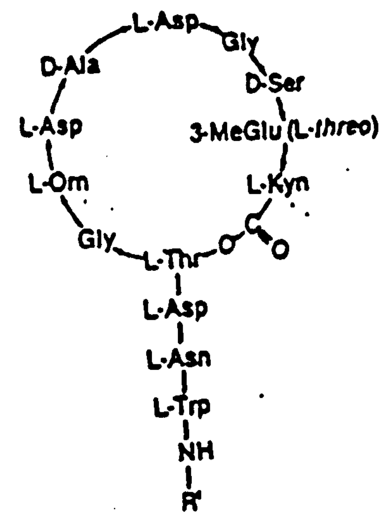
die eine spezifische Aktivität zwischen etwa 10 E/mg und etwa 11,25 E/mg aufweist, die eine Untereinheit mit 63 Kilodalton mit der folgenden aminoterminalen Sequenz aufweist: Ser-Asn-Ala-Tyr-Gly-Leu-Gly-Ala-Gln-Ala-Thr-Val-Asn-Gly-Ser-Gly-Met-Val-Leu-Ala-Asn-Pro-His-Phe-Pro-(Trp)-Gln --- Ala-Glu-(Arg)-Phe-Tyr und eine Untereinheit mit 18 Kilodalton mit der folgenden aminoterminalen Sequenz aufweist: His-Asp-Gly-Gly-Tyr-Ala-Ala-Leu-Ile-Arg-Arg-Ala-Ser-Tyr-Gly-Val-(Pro)-His-Ile-Thr-Ala-Asp-Asp-Phe und die bei der Deacylierung von Echinocandin B als Substrat eine optimale katalytische Aktivität bei etwa pH 6,0 bei etwa 60°C in 0,05 M KH_2PO_4 Puffer aufweist,

gekennzeichnet durch folgende Schritte 1) Erhitzen einer bei etwa pH 6,0 gepufferten wäßrigen Lösung der rohen Deacylase für etwa eine Stunde bei einer Temperatur von etwa 60°C, 2) Chromatographie des bei pH 6,0, 1,2 M KCl und 14 % $(NH_4)_2SO_4$ gepufferten hitzebehandelten Extrakts über ein hydrophobes Chromatographiematerial, 3) Fraktionierung des 95 % der Deacylaseaktivität enthaltenden Eluats von Schritt 2 durch $(NH_4)_2SO_4$ und Abtrennung der 10 % bis 36 % $(NH_4)_2SO_4$ Fraktion, 4) Gelfiltration dieser $(NH_4)_2SO_4$ Fraktion in 0,8 M KCl enthaltenden Puffer mit pH 6,0 und Vereinigen der Eluatsfraktion, die 90 % der Deacylaseaktivität enthält, 5) Chromatographie dieser Fraktionen in einem 0,05 M KCl Puffer mit pH 5,6 auf einem Kationenaustaucherchromatographiematerial und Elution dieses Chromatographiematerials mit einem linearen KCl Gradienten (0,05-0,5 M) in diesem Puffer, 6) Einstellen des die Deacylase enthaltenden Eluats von Schritt 5 auf 0,05 M KCl, Chromatographie dieses Eluats auf einem Farbstoffligandenchromatographiematerial in einem Puffer aus 0,05 M KH_2PO_4, und 0,05 M KCl mit pH 6,0 und Elution dieses Chromatographiematerials mit einem linearen KCl Gradienten (0,05 M-2,5 M), 7) Gelfiltration der vereinigten Eluatfraktionen von Schritt 6, die 80 % der Deacylaseaktivität enthalten in einem Puffer aus 0,05 M KH_2PO_4 und 0,2 M KCl mit pH 6,0 und 8) Einstellen des die Deacylase enthaltenden Eluats von Schritt 7 auf 0,04 M KCl bei pH 7, Chromatographie dieses Eluats auf einem Kationenaustauscherchromatographiematerial in einem Puffer aus 0,05 M KH_2PO_4 und 0,04 M KCl mit pH 7,0 und Elution der gereinigten Deacylase mit einem Stufenweisen KCl Gradienten (0,04-0,5-2 M).

2. Verfahren zur Deacylierung eines Cyclopeptids der Formel A oder B



A



B

gekennzeichnet durch Mischen dieses Cyclopeptids bei einer Temperatur zwischen etwa 25°C und etwa 75°C in einem wäßrigen Medium bei einem pH zwischen etwa 5 und etwa 7 mit der gereinigten Deacylase die durch das Verfahren nach Anspruch 1 hergestellt wurde, worin in Formel A R für Linoleoyl, Myristoyl oder Palmitoyl steht und in Formel B R' für Decanoyl, 8-Methyldecanoyl, 10-Methylundecanoyl oder 10-Methyldodecanoyl steht, unter Bereitstellung der Verbindung der Formel A oder B, worin R oder R' für Wasserstoff steht.

3. Verfahren nach Anspruch 2, worin die Temperatur bei etwa 55°C bis etwa 65°C gehalten wird.

4. Verfahren nach Anspruch 2, worin das wäßrige Medium ein anorganisches Salz enthält, das aus einem Alkalimetallchlorid oder einem Alkalimetallnitrat ausgewählt ist.

5. Verfahren nach Anspruch 4, worin das Salz Kaliumchlorid in einer Konzentration zwischen etwa 0,1 M bis etwa 3,0 M ist.

6. Verfahren nach Anspruch 2, worin in Formel A R für Linoleoyl steht.

7. Verfahren nach Anspruch 2, worin in Formel A R für Palmitoyl steht.

8. Verfahren nach Anspruch 2, worin in Formel B R' für Decanoyl steht.

9. Verfahren nach Anspruch 2, worin das wäßrige Medium Dimethylsulfoxid in einer Konzentration zwischen etwa 5 % und etwa 15 % enthält.

Revendications

Revendications pour les Etats contractants suivants : AT, BE, CH, DE, DK, FR, GB, GR, IT, LI, NL, SE

1. Echinocandine B désacylase sous forme purifiée, à savoir un hétérodimère de 81 kilodaltons possédant la composition d'aminoacides ci-après:

Aminoacides	Nombre de résidus par 81,000 daltons
Asp+Asn	74
Thr	51

(suite)

Aminoacides	Nombre de résidus par 81,000 daltons
Ser	83
Glu+Gln	45
Pro	42
Gly	85
Ala	79
Cys	10
Val	48
Met	5
Ile	25
Leu	53
Tyr	20
Phe	24
His	21
Lys	11
Arg	62
Trp	19

qui possède une activité spécifique entre environ 10 U/mg et environ 11,25 U/mg, qui possède une sous-unité de 63 kilodaltons possédant la séquence amino terminale ci-après:

Ser-Asn-Ala-Tyr-Gly-Leu-Gly-Ala-Gln-Ala-Thr-Val-Asn-
Gly-Ser-Gly-Met-Val-Leu-Ala-Asn-Pro-His-Phe-Pro-(Trp)-
Gln --- Ala-Glu-(Arg)-Phe-Tyr; et

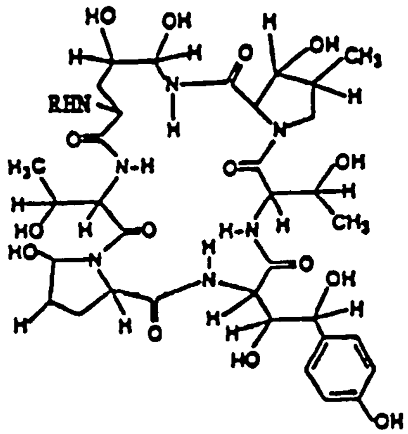
une sous-unité de 18 kilodaltons possédant la séquence amino terminale:

His-Asp-Gly-Gly-Tyr-Ala-Ala-Leu-Ile-Arg-Arg-Ala-Ser-Tyr-Gly-Val-(Pro)-His-Ile-
Thr-Ala-Asp-Asp-Phe, et

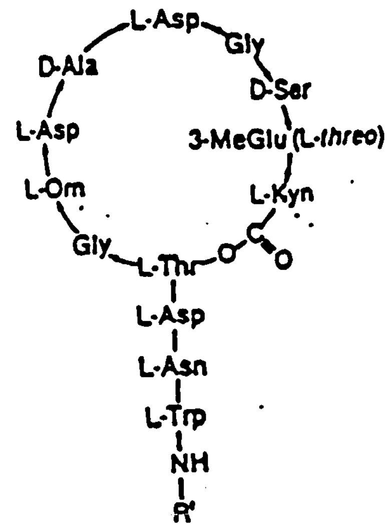
qui, dans la désacylation de l'échinocandine B comme substrat, manifeste une activité catalytique optimale à un pH d'environ 6,0 à une température d'environ 60°C dans un tampon de KH_2PO_4 0,05M.

2. Procédé pour préparer la désacylase purifiée selon la revendication 1, qui comprend les étapes consistant à: (1) chauffer pendant environ 1 heure, à une température d'environ 60°C, une solution aqueuse de désacylase brute dans un tampon dont le pH est d'environ 6,0; (2) chromatographier l'extrait ayant subi un traitement thermique, dans un tampon de pH 6,0, contenant KCl 1,2M et $(NH_4)_2SO_4$ à 14%, par-dessus un chromatogramme à interaction hydrophobe; (3) fractionner par $(NH_4)_2SO_4$ l'éluat contenant 95% de l'activité de désacylase de l'étape 3 et séparer les fractions contenant de 10% à 36% de $(NH_4)_2SO_4$; (4) filtrer sur gel lesdites fractions $(NH_4)_2SO_4$ dans un tampon de pH 6,0 contenant KCl 0,8M et combiner les fractions de l'éluat contenant 90% de l'activité de désacylase; (5) chromatographier lesdites fractions dans un tampon contenant KCl 0,05M, de pH 5,6, sur un chromatogramme d'échange de cations et éluer ledit chromatogramme avec un gradient linéaire de KCl (0,05-0,5M) dans ledit tampon; (6) régler l'éluat de l'étape 5 contenant la désacylase à KCl 0,05M, chromatographier ledit éluat sur un chromatogramme à ligand de coloration dans un tampon contenant KH_2PO_4 0,05M, pH 6,0, et KCl 0,05M, et éluer ledit chromatogramme avec un gradient linéaire de KCl (0,05M-2,5M); (7) filtrer sur du gel les fractions d'éluat rassemblées de l'étape 6 contenant 80% de l'activité de désacylase dans un tampon contenant KH_2PO_4 0,05M, pH 6,0, et KCl 0,2M; et (8) régler à KCl 0,04M à un pH de 7 l'éluat de l'étape 7 contenant la désacylase, chromatographier ledit éluat sur un chromatogramme d'échange de cations dans un tampon contenant KH_2PO_4 0,05M, pH 7,0, et KCl 0,04M, et éluer la désacylase purifiée avec un gradient progressif de KCl (0,04-0,5-2M).

3. Procédé pour désacyler un peptide cyclique répondant à la formule A ou B



A



B

qui comprend le fait de mélanger ledit peptide cyclique à une température entre environ 25°C et environ 75°C dans un milieu aqueux à un pH entre environ 5 et environ 7 avec la désacylase purifiée selon la revendication 1, dans lequel, dans la formule A, R représente un groupe linoléoyle, un groupe myristoyle ou un groupe palmitoyle, et dans la formule B, R' représente un groupe décanoyle, un groupe 8-méthyldécanoyle, un groupe 10-méthylundécanoyle ou un groupe 10-méthyldodécanoyle, pour obtenir le composé de formule A ou B dans lesquelles R ou R' représente un atome d'hydrogène.

4. Procédé selon la revendication 3, dans lequel on maintient une température d'environ 55°C à environ 65°C.

5. Procédé selon la revendication 3, dans lequel le milieu aqueux contient un sel inorganique choisi parmi un chlorure de métal alcalin ou un nitrate de métal alcalin.

6. Procédé selon la revendication 5, dans lequel le sel est le chlorure de potassium à une concentration entre environ 0,1M et environ 3,0M.

7. Procédé selon la revendication 3, dans lequel, dans la formule A, R représente un groupe linoléoyle.

8. Procédé selon la revendication 3, dans lequel, dans la formule A, R représente un groupe palmitoyle.

9. Procédé selon la revendication 3, dans lequel, dans la formule B, R' représente un groupe décanoyle.

10. Procédé selon la revendication 3, dans lequel le milieu aqueux contient du diméthylsulfoxyde en une concentration entre environ 5% et environ 15%.

Revendications pour l'Etat contractant suivant : ES

1. Procédé pour préparer de l'échinocandine B désacylase sous forme purifiée, à savoir un hétérodimère de 81 kilodaltons possédant la composition d'aminoacides ci-après:

Aminoacides	Nombre de résidus par 81,000 daltons
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(suite)

Aminoacides	Nombre de résidus par 81,000 daltons
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His	21
Lys	11
Arg	62
Trp	19

qui possède une activité spécifique entre environ 10 U/mg et environ 11,25 U/mg, qui possède une sous-unité de 63 kilodaltons possédant la séquence amino terminale ci-après:

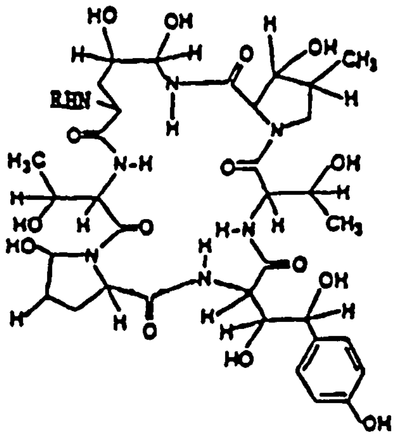
Ser-Asn-Ala-Tyr-Gly-Leu-Gly-Ala-Gln-Ala-Thr-Val-Asn-
Gly-Ser-Gly-Met-Val-Leu-Ala-Asn-Pro-His-Phe-Pro-(Trp)-
Gln — Ala-Glu-(Arg)-Phe-Tyr; et

une sous-unité de 18 kilodaltons possédant la séquence amino terminale:

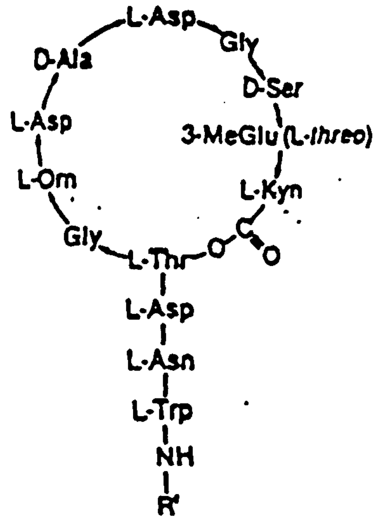
His-Asp-Gly-Gly-Tyr-Ala-Ala-Leu-Ile-Arg-Arg-Ala-Ser-Tyr-Gly-Val-(Pro)-His-Ile-
Thr-Ala-Asp-Asp-Phe, et

qui, dans la désacylation de l'échinocandine B comme substrat, manifeste une activité catalytique optimale à un pH d'environ 6,0 à environ 60°C dans un tampon de KH_2PO_4 0,05M, qui comprend les étapes consistant à: (1) chauffer pendant environ 1 heure, à une température d'environ 60°C, une solution aqueuse de désacylase brute dans un tampon dont le pH est d'environ 6,0; (2) chromatographier l'extrait ayant subi un traitement thermique, dans un tampon de pH 6,0, contenant KCl 1,2M et $(NH_4)_2SO_4$ à 14%, par-dessus un chromatogramme à interaction hydrophobe; (3) fractionner par $(NH_4)_2SO_4$ l'éluat contenant 95% de l'activité de désacylase de l'étape 3 et séparer les fractions contenant de 10% à 36% de $(NH_4)_2SO_4$; (4) filtrer sur gel lesdites fractions $(NH_4)_2SO_4$ dans un tampon de pH 6,0 contenant KCl 0,8M et combiner les fractions de l'éluat contenant 90% de l'activité de désacylase; (5) chromatographier lesdites fractions dans un tampon contenant KCl 0,05M, pH 5,6, sur un chromatogramme d'échange de cations et éluer ledit chromatogramme avec un gradient linéaire de KCl (0,05-0,5M) dans ledit tampon; (6) régler l'éluat de l'étape 5 contenant la désacylase à KCl 0,05M, chromatographier ledit éluat sur un chromatogramme à ligand de coloration dans un tampon contenant KH_2PO_4 0,05M, pH 6,0, et KCl 0,05M, et éluer ledit chromatogramme avec un gradient linéaire de KCl (0,05M-2,5M); (7) filtrer sur du gel les fractions d'éluat rassemblées de l'étape 6 contenant 80% de l'activité de désacylase dans un tampon contenant KH_2PO_4 0,05M, pH 6,0, et KCl 0,2M; et (8) régler à KCl 0,04M à un pH de 7 l'éluat de l'étape 7 contenant la désacylase, chromatographier ledit éluat sur un chromatogramme d'échange de cations dans un tampon contenant KH_2PO_4 0,05M, pH 7,0, et KCl 0,04M, et éluer la désacylase purifiée avec un gradient progressif de KCl (0,04-0,5-2M).

2. Procédé pour désacyler un peptide cyclique répondant à la formule A ou E



A



B

qui comprend le fait de mélanger ledit peptide cyclique à une température entre environ 25°C et environ 75°C dans un milieu aqueux à un pH entre environ 5 et environ 7 avec la désacylase purifiée selon la revendication 1, dans lequel, dans la formule A, R représente un groupe linoléoyle, un groupe myristoyle ou un groupe palmitoyle, et dans la formule B, R^1 représente un groupe décanoyle, un groupe 8-méthyldécanoyle, un groupe 10-méthylundécanoyle ou un groupe 10-méthyldodécanoyle, pour obtenir le composé de formule A ou B dans lesquelles R ou R^1 représente un atome d'hydrogène.

3. Procédé selon la revendication 2, dans lequel on maintient une température d'environ 55°C à environ 65°C.

4. Procédé selon la revendication 2, dans lequel le milieu aqueux contient un sel inorganique choisi parmi un chlorure de métal alcalin ou un nitrate de métal alcalin.

5. Procédé selon la revendication 4, dans lequel le sel est le chlorure de potassium à une concentration entre environ 0,1M et environ 3,0M.

6. Procédé selon la revendication 2, dans lequel, dans la formule A, R représente un groupe linoléoyle.

7. Procédé selon la revendication 2, dans lequel, dans la formule A, R représente un groupe palmitoyle.

8. Procédé selon la revendication 2, dans lequel, dans la formule B, R' représente un groupe décanoyle.

9. Procédé selon la revendication 2, dans lequel le milieu aqueux contient du diméthylsulfoxyde en une concentration entre environ 5% et environ 15%.